Original Paper

Protective Effects of Cerium Oxide Nanoparticles on MC3T3-E1 Osteoblastic Cells Exposed to X-Ray Irradiation

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Key Words

X-ray irradiation • Cerium oxide nanoparticles • MC3T3-E1 cells • Oxidative stress • Micronucleus • Mineralization

Abstract

Background/Aims: Exposure to ionizing radiation can result in bone damage, including decreased osteocyte number and suppressed osteoblastic activity. However, molecular mechanisms remain to be elucidated, and effective prevention strategies are still limited. This study was to investigate whether cerium oxide nanoparticles (CeO₂ NP) can protect MC3T3-E1 osteoblast-like cells from damaging effects of X-ray irradiation, and to study the underpinning mechanism(s). **Methods:** MC3T3-E1, a osteoblast-like cell line, was exposed to X-ray irradiation and treated with different concentration of CeO₂ nanoparticles. The micronucleus frequency was counted under a fluorescence microscope. Cell viability was evaluated using MTT assay. The effects of irradiation and CeO₂ nanoparticles on alkaline phosphatase activity and MC3T3-E1 mineralization were further assayed. **Results:** We found that the ratio of micronuclei to binuclei was dose-dependently increased with X-ray irradiation (from 2 to 6 Gy), but diminished with the increased concentration of CeO₂ NP treatment (from 50 to 100 nM). Exposure to X-rays (6 Gy) decreased cell viability, differentiation and the mineralization, but CeO₂ NP treatment (100 nM) attenuated the deteriorative effects of irradiation. Both intracellular reactive oxygen species (ROS) production and extracellular H₂O₂ concentration were increased after X-ray irradiation, but reduced following CeO₂ NP treatment. Similar to irradiation, exposure to H₂O₂ (10 μM) elevated the frequency of micronuclei and diminished cell viability and mineralization, while these changes were ameliorated following CeO₂ NP treatment. **Conclusions:** Taken together, our findings suggest that CeO₂ nanoparticles exhibit astonishing protective effects on irradiation-induced osteoradionecrosis in MC3T3-E1 cells, and the protective effects appear to be mediated, at least partially, by reducing oxidative stress.

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Introduction

Ionizing irradiation is an occupational health risk factor in space travel (astronauts), nuclear plants and medical imaging. For example, exposure to high-dose ionizing irradiation can cause deleterious effects on bone tissue, and osteoradionecrosis is often seen in cancer patients treated with ionizing irradiation [1, 2]. In addition, other serious bone complications, including loss of bone mass, bone fracture and sclerosis, have been reported [3, 4]. Increased osteoblastic damage appears to play an important role in the reduced bone mineral density following irradiation [5]. It has been reported that ionizing irradiation can lead to the impeded osteoblast proliferation and differentiation, cell-cycle arrest, and even apoptosis [2, 6, 7]. However, the exact molecular mechanisms underlying irradiation-induced bone injury remain unclear, and therefore the effective radio-protectants are still limited.

Recently, the possible application of nanomaterials in biomedical fields has been drawn much attention, owing to their unique physicochemical and bioactive properties [8]. In particular, cerium oxide (CeO₂) nanoparticles are one of the most interesting nanomaterials. Its possible protection against retinal neurodegeneration, and anti-inflammatory and antioxidant activities have been recently studied [9-12]. Cerium oxide is a rare earth oxide material and exhibits the ability to cycle between the Ce⁺³ (fully reduced) and Ce⁺⁴ (fully oxidized) states [13]. Therefore, CeO₂ nanoparticles may mimic the function of superoxide dismutase (SOD), catalase, and free radical scavenger [13-17], and can protect against the pathological progression of cardiac dysfunction and remodeling [11]. Other studies suggested that CeO₂ nanoparticles can prolong the cell longevity and reduce UV light-induced cell injury [12]. Whether CeO₂ nanoparticle treatment can diminish the osteoblast injury caused by X-ray radiation is currently unclear.

On basis of the facts that CeO₂ nanoparticles may possess antioxidant properties [13-17], we hypothesize that CeO₂ nanoparticles can decrease osteoradionecrosis and hence protect bone cells from irradiation. In this study, we investigated the effects of X-ray irradiation and CeO₂ treatment on MC3T3-E1 cells *in vitro*. Using hydrogen peroxide (H₂O₂) to mimic increasing oxidative condition, we also studied the possible role of oxidative stress in X-ray induced MC3T3-E1 cell injury. Our data suggest that CeO₂ nanoparticle treatment is effective in diminishing DNA damage and oxidative stress, and reducing osteoblast injury following X-ray irradiation.

Materials and Methods

Materials

Cerium (IV) oxide dispersion nanoparticles (#643009; particle size (<25 nm) determined by the Brunauer–Emmett–Teller method), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), cytochalasin B, β-glycerophosphate, L-ascorbic acid, and MTT assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydroethidium (DHE) and Amplex Red Hydrogen Peroxide Assay kit were from Molecular Probes (Eugene, OR, USA). Alkaline phosphatase (ALP) activity assay kit was from BioVision Inc. (Milpitas, CA, USA) and osteogenesis quantitation kit was from EMD Millipore (Billerica, MA, USA). Hydrogen peroxide (H₂O₂) was purchased from Acros Organics (Fair Lawn, NJ, USA). MC3T3-E1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA).

Cell culture and treatment

MC3T3-E1 is an osteoblastic cell line established from C57BL/6 mouse calvaria. This cell line has a similar behavior to primary osteoblasts, and provides a good model for *in vitro* study [18]. Cells were cultured in α-MEM medium consisting of 10% fetal bovine serum (FBS), and maintained in an ambient of 95% air and 5% CO₂ at 37°C, as described previously [19]. Cell medium was changed every three days. When MC3T3-E1 cells reached 80% confluence, they were detached by treatment with 0.05% trypsin, and re-plated for experiments. Cells used in experiments were between 8 and 10 passages. The cells used in differentiation and mineralization experiments were cultured in α-MEM medium consisting of 10% FBS, 5 mM β-glycerophosphate, and 50 μg/mL L-ascorbic acid after the cells reaching 80% confluence, as described

previously [20]. The cells were treated with CeO₂ nanoparticles (50 or 100 nM) prior to the exposure to 6 Gy X-ray irradiation with an X-RAD 320 system (North Branford, CT, USA). In H₂O₂ experiments, the treatment was performed by adding H₂O₂ (10 µM) every day along with the medium change every three days, and the protective effects of CeO₂ nanoparticles (100 nM) were evaluated.

Micronuclei (MN) scoring

The formation of MN was evaluated by the cytokinesis block MN technique developed by Fenech and Morley [21, 22]. In brief, MC3T3-E1 cells were exposed to X-ray irradiation (2, 4 or 6 Gy) or H₂O₂ after treatment with or without CeO₂ nanoparticles. At the 0, 24th, and 48th h after the irradiation, or on the 6th day after H₂O₂ exposure, the cells were incubated in the medium containing 2.5 mg/mL cytochalasin B for additional 48 h, rinsed with PBS for three times, and fixed in methanol / acetic acid (9:1, v/v) for 20 min. The air-dried MC3T3-E1 cells were stained with DAPI (10 µg/mL), and the number of micronuclei in the binucleated cells was counted under a fluorescent microscope Carl Zeiss Axioplan-2 [23, 24]. The ratio of micronuclei to binucleated cells (1000 binucleated cells) was calculated. At least 1000 binucleated cells were scored for each sample in three separate experiments.

Cell viability assay

After X-ray irradiation or H₂O₂ exposure along with or without CeO₂ nanoparticles treatment, the cell viability was measured with MTT assay kit as described previously [25, 26]. In brief, the MC3T3-E1 cells were incubated with the MTT reagent at 37°C for 4 h, and then the formed formazan crystals was dissolved in 200 µL of dimethyl sulphoxide (DMSO). Optical density (OD) was detected at 490 nm using the microplate scanning spectrophotometer (Bio-Tek, Winooski, VT, USA).

ALP activity assay

The MC3T3-E1 cells were cultured for 7 days after irradiation along with or without CeO₂ treatment, rinsed with PBS for three times, and lysed in CellLytic M cell lysis reagent. Protein concentration in the supernatant was measured using a Pierce 660 nm protein assay reagent, and the ALP activity was determined by ALP activity assay kit. ALP activity in the supernatant was normalized to its protein concentration and expressed as units per gram protein.

Mineralization assay

The degree of mineralization (calcium-rich deposits) in MC3T3-E1 cells was determined in 6-well plates by Alizarin Red Staining (ARS) according to the manufacturer's protocols. In brief, on the 21st day after X-ray irradiation or H₂O₂ incubation along with or without CeO₂ nanoparticles treatment, MC3T3-E1 cells were fixed with 4% formaldehyde, washed thoroughly with deionized H₂O, and then incubated with ARS solution for 20 min. The mineral deposits were observed and the images were captured under a light microscopy. The ARS quantitative analysis was performed by determining OD values at 405 nm according to the manufacturer's protocols.

Dihydroethidium staining

At the 24th h after X-ray irradiation, the intracellular reactive oxygen species (ROS) levels were assessed by dihydroethidium staining as described previously [24, 27]. In brief, at the end of treatment, the cells were incubated with 10 mM of dihydroethidium for 1 h at 37°C. After washing with PBS, the fluorescence signal was captured under a fluorescence microscope Carl Zeiss Axioplan-2. The mean fluorescence intensity per nucleus was quantified using Image J software. At least 100 nuclear per sample were evaluated at 400× magnification.

Hydrogen peroxide assay

MC3T3 cells were cultured in the 24-well plates and immediately exposed to 6 Gy X-rays after CeO₂ nanoparticles treatment. At the 0 and 3rd h after irradiation, culture medium was collected and medium H₂O₂ concentrations were measured using Amplex Red Hydrogen Peroxide Assay kit according to the manufacturer's protocols. After incubation with Amplex Red reagents at room temperature for 30 min in the dark, fluorescence intensity was detected at 560 nm excitations and 590 nm emissions using the microplate scanning spectrophotometer (Bio-Tek, Winooski, VT, USA).

Statistical analysis

Each experiment was repeated independently at least three times. Data were expressed as mean $\pm$ standard error of mean. Multiple comparisons were performed using a one-way ANOVA and the Tukey post hoc test as appropriate. A $P \leq 0.05$ was considered as statistically significant.

Results

Effects of CeO₂ nanoparticles on micronuclei formation in X-ray irradiated MC3T3-E1 cells

To determine the effect of X-ray irradiation on cellular chromosome breaks, the number of cells containing micronuclei in 1000 binucleated cells was counted under a fluorescence microscope. Compared to the control, irradiation dose-dependently increased the frequency of micronuclei in MC3T3-E1 cells (from 2 to 6 Gy, Fig. 1A). The micronuclei frequency at all three irradiation doses reached the peak at the 24th h after the irradiation (Fig. 1A). Importantly, the elevation of micronuclei frequency was significantly attenuated after treatment with CeO₂ nanoparticles at 50 or 100 nM (Fig. 1B).

Effects of CeO₂ nanoparticles on the cell viability in X-ray irradiated MC3T3-E1 cells

The effects of irradiation and CeO₂ nanoparticle treatment on MC3T3-E1 cell growth were quantified using MTT assay. As shown in Fig. 2, X-ray irradiation (6 Gy) caused a significant decrease of cell viability in MC3T3-E1 cells when compared with the control, while cell viability was improved by 100 nM CeO₂ nanoparticles on the 4th day after the irradiation (Fig. 2). No significant difference was observed among four groups on the first day after the irradiation (Fig. 2).

Effects of CeO₂ nanoparticles on ALP activity in X-ray irradiated MC3T3-E1 cells

Alkaline phosphatase activity was determined on the 7th day after X-ray irradiation and CeO₂ nanoparticle treatment. Compared to the control group, ALP activity was significantly

Fig. 1. Effects of X-ray irradiation and CeO₂ nanoparticle treatment on the micronuclei formation. (A) The representative images of binuclei (control) and micronuclei (6 Gy) (Left panel), and the ratio of micronuclei (MN)/1000 binucleated cells in MC3T3-E1 cells exposed to different X-ray irradiation dose and at time points as indicated (Right panel). (B) The ratio of micronuclei/1000 binucleated cells in MC3T3-E1 cells treated with CeO₂ nanoparticles (50 and 100 nM) prior to the exposure to 6 Gy X-ray irradiation. Values are mean $\pm$ SEM of 3 independent experiments. abc: Groups without the same letter are significantly different ($P \leq 0.05$). Scale bar: 10 μ m.

Fig. 2. Effects of CeO₂ nanoparticles on the cell viability in X-ray irradiated MC3T3-E1 cells. Top panel: Representative images of cell morphology. Bottom panel: Cell viability as determined with MTT assay. Values are mean ± SEM of 3 independent experiments. abc: Groups without the same letter are significantly different (P ≤ 0.05). Scale bar: 50 μm.

decreased in the irradiation and two CeO₂ treatment groups (Fig. 3). However, there was no statistically significant difference between the irradiation and CeO₂ treatment groups (Fig. 3).

Effects of CeO₂ nanoparticles on the mineralization in X-ray irradiated MC3T3-E1 cells

Cellular mineralization (calcium-rich deposits) was performed on the 21st day after X-ray irradiation and CeO₂ nanoparticle treatments. ARS staining showed that the number of mineralized nodules was remarkably decreased in the irradiation group when compared with the control group, but was improved in the CeO₂ treatment groups (Fig. 4).

Fig. 3. Effects of CeO₂ nanoparticles on the alkaline phosphatase activity in X-ray irradiated MC3T3-E1 cells. ALP activity was determined on the 7th day after irradiation and CeO₂ nanoparticle treatment. Values are mean ± SEM of 3 independent experiments. ab: Groups without the same letter are significantly different (P ≤ 0.05).

Fig. 4. Effects of CeO₂ nanoparticles on the mineralization in X-ray irradiated MC3T3-E1 cells. The calcium-rich deposits in MC3T3-E1 were detected on the 21st day after the irradiation. Top panel: Representative images of Alizarin Red staining. Bottom panel: Quantitative analysis of Alizarin Red staining. Values are mean ± SEM of 3 independent experiments. abcd: Groups without the same letter are significantly different (P ≤ 0.05). Scale bar: 200 μm.

Fig. 5. Effects of CeO₂ nanoparticles on intracellular ROS production and extracellular H₂O₂ concentration in X-ray irradiated MC3T3-E1 cells. (A) Intracellular ROS was detected by dihydroethidium (DHE) staining at the 24th h after the treatments. Top panel: Representative images of dihydroethidium staining. Bottom panel: Quantitative analysis of dihydroethidium staining. (B) Extracellular H₂O₂ were measured by Amplex Red assay. Values are mean ± SEM of 3 independent experiments. abc: Groups without the same letter are significantly different (*P* ≤ 0.05). Scale bar: 50 μm.

Effects of CeO₂ nanoparticles on intracellular ROS and extracellular H₂O₂ in X-ray irradiated MC3T3-E1 cells

Dihydroethidium, a ROS indicator, intercalates within oxidized DNA and stains the nucleus a bright fluorescent red [28]. Compared to the control, the fluorescence intensity was increased in the irradiated cells, but significantly reduced following CeO₂ nanoparticles treatment (Fig. 5A). Similarly, compared to the control, H₂O₂ concentration in the medium at the 3rd h (normalized to that at 0 h) after irradiation was significantly increased in the irradiated cells, but CeO₂ treatment restored the H₂O₂ concentration to that comparable to the control group (Fig. 5B).

Effects of CeO₂ nanoparticles on H₂O₂-induced damages in MC3T3-E1 cells

To study the possible role of oxidative stress on irradiation-induced micronuclei formation and inhibition of cell viability and mineralization, H₂O₂ was used to mimic the oxidative stress condition. Similar to X-ray irradiation, the ratio of micronuclei to the binucleated cells was increased in MC3T3-E1 cells exposed to H₂O₂, but significantly attenuated after treatment with CeO₂ nanoparticles (100 nM, Fig. 6A). Exposure to H₂O₂ caused a decrease of cell viability in MC3T3-E1 cells, while it was ameliorated after treated with 100 nM CeO₂ nanoparticles (Fig. 6B). The number of mineralized nodules in MC3T3-E1 cells exposed to H₂O₂ was also remarkably decreased when compared to the control, but was increased following CeO₂ nanoparticle treatment (Fig. 6C).

Discussion

Accumulating evidences from occupational, environmental, experimental and clinical investigations suggested that ionizing radiation can cause decreases of osteoblast proliferation, differentiation and mineralization, which if allowed to proceed unchecked

Fig. 6. Effects of CeO₂ nanoparticles on H₂O₂-induced damages in MC3T3-E1 cells. (A) Micronuclei of MC3T3-E1 cells were counted under the fluorescence microscope on the 6th day after treatments. (B) Cell viability of MC3T3-E1 cells was measured with MTT assay on the 3rd day after the treatments (Scale bar: 50 μ m). (C) The mineralization (calcium-rich deposits) of MC3T3-E1 cell was determined using the osteogenesis quantitation kit on day 21st after the treatments (Scale bar: 200 μ m). Values are mean $\pm$ SEM of 3 independent experiments. abc: Groups without the same letter are significantly different ($P \leq 0.05$).

can lead to osteoradionecrosis and bone fracture [6, 29-32]. However, the effective protection strategies are still limited. In this study, we observed that CeO₂ nanoparticle treatment can decrease micronuclei formation (chromosome breaks) but increase cell viability and mineralization in MC3T3-E1 cells exposed to X-ray irradiation. We also found that CeO₂ nanoparticles could reduce the increase of intracellular ROS production and extracellular H₂O₂ accumulation in X-ray irradiated cells. To further investigate whether the protective effects of CeO₂ nanoparticles on MC3T3-E1 cells are mediated by inhibiting oxidative stress, we demonstrated that CeO₂ nanoparticle is able to attenuate the increase of micronuclei formation and the decrease of cell viability and mineralization in MC3T3-E1 cells treated with 10 μ M of H₂O₂. These data support that CeO₂ nanoparticles exhibit protective effects in attenuating oxidative stress and cellular damages induced by X-ray irradiation in MC3T3-E1 cells.

Ionizing irradiation is a common occupational hazard to those occupations in space travel, nuclear plants, medical imaging and welding. In addition, radiation therapy in cancer patients may also lead to irradiating damages to normal tissues [1]. For example, studies have shown that high dose of ionizing irradiation can lead to osteoradionecrosis, loss of bone mass and bone fracture [3, 4, 33]. Therefore, developing effective radio-protectants is

more valuable. Unfortunately, the available substances that have exhibited radio-protective properties are very limited [34-36]. Since living cells consist of nearly 70% water, ionizing irradiation can lead to the formation of free radicals, as the radiation interacts with water within the cell [22, 37, 38]. Therefore, we expect that increased intracellular ROS, if present in X-ray irradiated osteoblastic cells, could damage to macromolecules, such DNAs, proteins, and lipids. To attest the notion, MC3T3-E1 osteoblast-like cells were exposed to different doses of X-ray irradiation (2, 4 or 6 Gy, respectively). We found that after 24 hours exposure of MC3T3-E1 cells to 6 Gy of X-ray, intracellular ROS levels were significantly increased as detected by the dihydroethidium (Fig. 5A). Similarly, the extracellular H₂O₂ level was also elevated in the irradiated cells (Fig. 5B). To further examine whether irradiation-induced excess ROS production was associated with intracellular DNA damage and chromosome breaks, we examined the formation of micronuclei in MC3T3-E1 cells after exposure to X-rays, and found that X-ray irradiation dose-dependently increased the ratio of micronuclei to binuclei, and the ratio reached the peak approximately at the 24th h after the irradiation (Fig. 1A). To further assure the role of oxidative stress in X-ray irradiation, hydrogen peroxide was used to mimic oxidative stress condition, and increased chromosome fragmentation was confirmed in H₂O₂-treated MC3T3-E1 cells (Fig. 6A).

To further evaluate the role of oxidative stress and the damaging effects of X-ray irradiation on bone cell growth and differentiation, we examined bone cell viability, ALP activity (as the early osteoblastic phenotypic marker [39, 40]) and mineralization (formation of mineralized nodules [41]). Consistent with the increased intracellular ROS production and extracellular H₂O₂ concentration (Fig. 5) and micronuclei frequency (Fig. 1), we found that X-ray irradiation inhibited cell viability, and decreased intracellular ALP activity and mineralization in MC3T3-E1 cells (Fig. 2-4). In parallel with increased chromosome fragmentation (Fig. 6A), our data showed that both cell viability and mineralization of MC3T3-E1 cells were diminished by H₂O₂ (Fig. 6B-C). These data together support strongly the critical role of oxidative stress in osteoradionecrosis associated with irradiation.

Cerium oxide nanoparticles have recently been shown to possess great pharmacological potentials in protection against retinal neurodegeneration, anti-inflammatory and antioxidant activities [9-12]. As a rare earth oxide material, cerium oxide exhibits amazing ability to cycle between the Ce⁺³ (fully reduced) and Ce⁺⁴ (fully oxidized) states [13]. Therefore, CeO₂ nanoparticles may act as superoxide dismutase (SOD) and catalase mimetics [14, 15], and free radical scavenger [13, 16, 17]. Recent data has suggested that CeO₂ nanoparticles can prolong the cell longevity and reduce UV light-induced cell injury by over 60% in cultured brain cells [12]. However, no study has been reported that whether CeO₂ nanoparticle treatment can diminish the osteoblast injury after exposure to X-ray irradiation. As shown in Fig. 5, we demonstrated that CeO₂ nanoparticles can diminish the production of ROS in X-ray irradiated MC3T3-E1 cells. The anti-oxidative properties of CeO₂ nanoparticles were able to reduce micronuclei formation (Fig. 1 and 6A), and increase cell viability (Fig. 2 and 6B) and mineralization (Fig. 4 and 6C) in both X-ray irradiation and H₂O₂-treated cells. These findings, for the first time, demonstrate the protective effects of CeO₂ nanoparticles on osteoblastic cells, and the underpinning mechanism, at least partially, by diminishing irradiation-induced oxidative stress.

In summary, our results show that CeO₂ nanoparticles exhibit protective effects on MC3T3-E1 cells against irradiation or H₂O₂-induced damages, including formation of micronuclei (chromosome fragmentation), and impaired cell viability and mineralization. The radioprotective effects of CeO₂ nanoparticles are mediated, at least in part, by its anti-oxidative activity. Future *in vivo* studies are necessary to attest the protective effects of CeO₂ nanoparticles on bone structure and function and possibly clinical application.

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Disclosure Statement

All the authors declared no competing financial interests.

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