

Harnessing cerium oxide nanoparticles to protect normal tissue from radiation damage

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Abstract: At the onset of radiation exposure, free radicals are formed through ionizing reactions that are then capable of destroying normal tissues. While cells release a level of protective molecules, such as glutathione and metallothioneine, they are not capable of blocking all damage, thus resulting in the death of normal tissues and therefore, we must continue to develop strategies to protect normal tissues from radiation-induced damage. One such strategy is the development of radiation protectors. Several compounds have been described, but Amifostine (Ethyol), whose active free thiol metabolite WR-1065 has been shown to prevent both radiation-induced cell death and mutagenesis while facilitating the repair of normal cells remains the only agent currently in clinical use. Major limitations to the clinical use of Amifostine are its short half-life, daily dosing requirements, toxicity based on route of administration, and its cost. Recent studies have shown the effects of engineered cerium oxide (CeO_2) nanoparticles for protection against radiation-induced damage in a variety of tissue types. The role of nanoparticles as radioprotectants is a cutting-edge development in decades of scientific interest regarding the protection of normal cells and tissues from radiation. The chemistry of engineered CeO_2 nanoparticles supports a potential role as a biological free radical scavenger or antioxidant. The work presented in this review article will address the effectiveness of CeO_2 nanoparticles in radioprotection in a variety of cells and in animal models during radiation exposure which will encourage the development of innovative and new approaches to radiation protection, using nanotechnology.

Key Words: Radiation protection; normal tissues; cerium oxide (CeO_2); nanoparticles; free radicals



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Introduction

Free radicals are formed through ionizing reactions, such as the photoelectric, Compton and Auger effects. These free radicals react with DNA and RNA, causing molecular alterations, improper segregation of chromosomes during mitosis, and radiation-induced mitotic death (mitotic catastrophe) (1,2). Furthermore, radiation-induced cellular oxidative damage is initiated by the generation of reactive oxygen species (ROS), which are known to change the oxidative status of cells, resulting in changes in mitochondrial function and activation/inactivation of various proteins involved in the apoptosis (cell death) process (3). When healthy (normal) cells are exposed to radiation, they

ameliorate the damaging effect of free radicals by the release of innate protective molecules such as superoxide dismutase (SOD), glutathione, and metallothioneine, which increase and intensify DNA repair mechanisms (3). Nonetheless, while these protective and repair mechanisms for cells are efficient, they are not capable of blocking all of the damage, which ultimately leads to normal tissue death.

In an effort to combat the harmful effects of radiation, various free radical scavengers have been tested for their ability to protect normal cells and tissues. Free radical scavengers such as Amifostine, Vitamin E, ascorbate, carotenes, melatonin and lipoic acid derivatives are the subject of many recent reviews (4). However, many of

these free radical scavengers were found to have limited success due to short half-lives (hours or even minutes), lack of penetration to the site of radical production, and daily dosing requirements. This report discusses a novel approach for the protection of normal cells against radiation-induced cell damage by using cerium oxide (CeO₂) nanoparticles.

Most recently, CeO₂ nanoparticles have been tested for their ability to serve as free radical scavengers (5-7) to render protection against chemical, biological and radiological insults that promote the production of free radicals. The chemistry of engineered CeO₂ nanoparticles supports a potential role as a biological free radical scavenger or antioxidant. It was suggested that the unique structure of CeO₂ nanoparticles, with respect to valence and oxygen defects, promotes cell longevity and decreases toxic insults by virtue of its antioxidant properties that occurs when the nanoparticles enter the cells (8), prevent the accumulation of ROS and thereby preventing the activation of the apoptotic response and death of the cells (5).

In this report, CeO₂ nanoparticles are shown to confer protection against radiation-induced cell damage *in vitro* and *in vivo*, suggesting that CeO₂ nanoparticles are an effective radioprotectant for normal tissues.

Radiotherapy side effects

No cancer treatment is without side effects. Following radiotherapy, many patients experience side effects such as mild neutropenia, swelling or pain, and telangiectasia (a sunburn-type appearance of the skin); however these early side effects usually disappear within several weeks. Early side effects occur in rapidly proliferating tissues, and are generally not dose-limiting factors, and have minimal long term impact upon the quality of life (QOL) of the patient. Of far greater concern, is the emergence of late-reacting tissue damage in organs such as the lungs, skin and spinal cord; radiation damage to such tissues manifests itself weeks to months after the completion of therapy. These severe normal tissue reactions cause extensive discomfort to the affected individuals and limit the radiation dose that can be delivered to the entire patient population.

CeO₂ nanoparticles as radioprotectants

Nanotechnology is a multidisciplinary field that involves the design and engineering of objects <100 nanometers (nm) in size. A new generation of free radical scavengers is nanoparticles. The role of nanoparticles as radioprotectants

is a cutting-edge development addressing decades of scientific interest regarding the protection of normal cells and tissues from radiation. The chemistry of engineered CeO₂ nanoparticles supports a potential role as a biological free radical scavenger or antioxidant. Current studies highlighted in this chapter suggest that nanoparticles may be a therapeutic regenerative material that will scavenge ROS that are responsible for radiation-induced cell damage.

CeO₂ nanoparticles in biological applications

While there are some concerns about the toxicity of nanoparticles, there are very few reports regarding the biologically detrimental effects of CeO₂ nanoparticles. In an article published recently in Toxicology, Park *et al.* conclude that CeO₂ nanoparticles (15-45 nm; 5-40 µg/mL) induced oxidative stress and cell death in cultured human lung epithelial cells (9). It is important to note that these particles are significantly larger than the nanoparticles used in the experiments discussed because the size of a nanoparticle affects the free radical scavenging ability of the particle by modifying the ratio of cerium (III) to cerium (IV). Furthermore, Park *et al.* exposed the cells to CeO₂ nanoparticles doses ~1,000 times the effective radioprotective dose was recently published (6).

Need for better radioprotective compound

Free radical scavengers such as Amifostine, Vitamin E, ascorbate, carotenes, melatonin and lipoic acid derivatives possess few active sites per molecule. A more recently investigated antioxidant, C60, may be able to scavenge a comparatively more number of radicals than the currently available antioxidants (10). But, due to the limited number of free radical scavenging sites, repeated dosing is required to replace molecular species that were utilized in free radical reduction. However, CeO₂ nanoparticles offer many active sites for free radical scavenging due to their large surface to volume ratio and, more importantly, due to their mixed valence states for unique redox chemistry. A recent article reports SOD mimetic activity of CeO₂ (11). Additionally, the free radical scavenging property of CeO₂ nanoparticles is regenerative (6) which is not the case for other antioxidants. It is believed that due to the chemical nature of CeO₂ nanoparticles, there is an auto-regenerative reaction cycle ($\text{Ce}^{3+} \rightarrow \text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$) continuing on the surface of ceria nanoparticles and is thought to be the current mechanism by which it provides the material with an

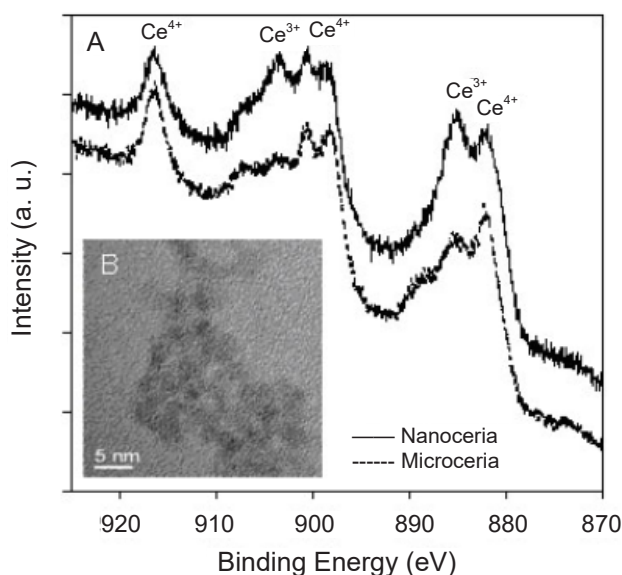


Figure 1 Characterization of CeO₂ nanoparticles. A. X-ray photoelectron spectroscopy (XPS) spectra indicates high concentration of Ce³⁺ in CeO₂ compared to microceria particles. Peaks at 882.1 and 886 eV correspond to Ce⁴⁺ and Ce³⁺ peaks. Peaks at 918 eV correspond to satellite peaks indicating the presence of Ce⁴⁺ peak; B. High resolution transmission electron microscopy (HRTEM) image of the synthesized particles indicating the particle size of 3-5 nm with fluorite lattice structure. With permission from Baker C.H. 2009. Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. *Nanomedicine* 5:225-31

unprecedented free radical scavenging ability (Figure 1).

CeO₂ nanoparticles exhibit in vitro free radical scavenging ability

The chemistry of engineered CeO₂ nanoparticles supports their potential role as free radical scavengers, antioxidants, in biological systems (10). It was suggested that the unique surface chemistry of CeO₂ nanoparticles, with respect to valence and oxygen defects, decreases oxidative insults by virtue of its antioxidant properties and promotes cell longevity. Thus far, studies have shown that CeO₂ nanoparticles enter mammalian cells (8), decrease the accumulation of ROS, and prevent the activation of the ROS-induced apoptosis (5). Since cells produce ROS after being exposed to radiation (12), the antioxidant capability of CeO₂ nanoparticles has been suggested as the key mechanism by which CeO₂ nanoparticles confers radioprotection (6). Furthermore, a study concluded that

CeO₂ nanoparticles exhibited SOD-mimetic activity (12). Results supporting the antioxidant properties of CeO₂ nanoparticles is mounting, and many studies suggest that these nanoparticles act as free radical scavengers (6,7,13) and may render protection against chemical insults that promote the production of free radicals (14). Thus, it has been proposed that CeO₂ nanoparticles may confer radioprotection by scavenging the free radical produced during radiotherapy (6).

CeO₂ nanoparticles protect mice from total body irradiation (TBI)

Balb-C mice were randomized into 2 groups (n=10). Group 1 was injected with saline (control group). Group 2 received a total CeO₂ nanoparticles dose of 0.005 mg/kg. On day 5, all animals received 12.5 Gy of X-ray radiation. No animals died in the CeO₂ nanoparticles group during the first 60 days post irradiation. In sharp contrast, 20% of the control animals died (Figure 2A). During the experiment we observed that many of the control animals appeared exhibited skin desquamation, while the CeO₂ nanoparticles-treated animals had little skin damage (Figure 2B). These results suggest that CeO₂ nanoparticles are able to protect mice from a single dose of radiation, and support CeO₂ nanoparticles's role as a radioprotectant (15).

CeO₂ nanoparticles is well-tolerated in athymic mice

To investigate the acute toxicity of CeO₂ nanoparticles, athymic nude mice were randomized into five groups. Each group received a total nanoparticle dose in the range of 0 (saline), 0.135, 1.35, 13.5, or 135 mg/kg. The mice were observed over a three-week period. No mice died or experienced notable side effects during the treatment. At the end of the treatment, the mice were sacrificed. During necropsy no abnormal pathologies were observed. This indicates that CeO₂ nanoparticles are well-tolerated in mice up to 3 million times the effective dose. Therefore, it was suggested that CeO₂ nanoparticles causes limited toxicity and side effects in mice (15).

Applications to areas of health and disease

When biological systems are under high energy exposure ROS are produced at high levels and cellular components can be damaged. These ROS can be used by biological systems as a defense mechanism against microorganisms and

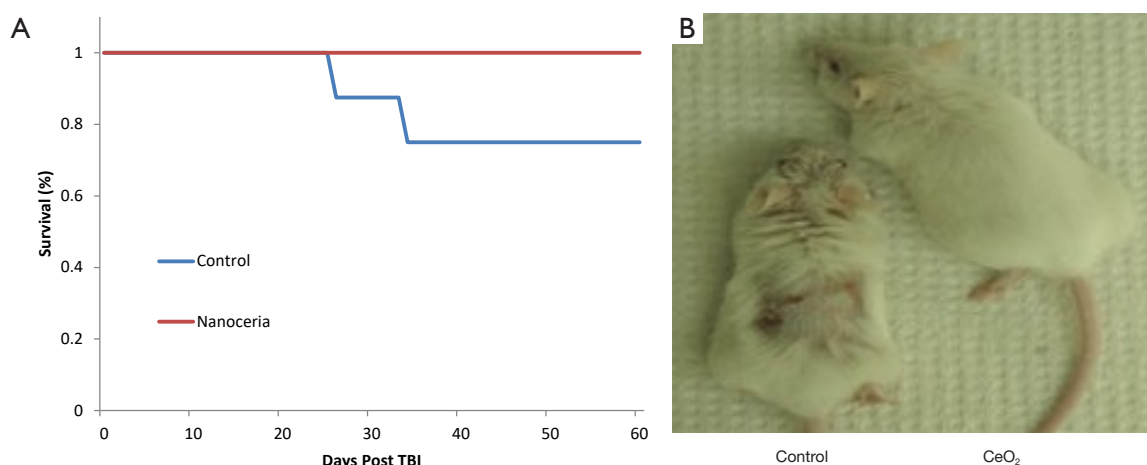


Figure 2 CeO₂ Nanoparticles protect mice from total body irradiation. A. Survival curve; B. Mice treated with CeO₂ nanoparticles had significantly less skin desquamation than untreated mice (control) 26 days after total body irradiation (12.5 Gy). *Unpublished data from Baker; C.H*

can act as signal transduction and transcription agents in development, stress responses, and programmed cell death. Oxidative stress arises from the strong cellular oxidizing potential of excess ROS, or free radicals. In addition, elevated levels of oxidative damage are related to increased risks for cataracts, cardiovascular disease, and cancer.

Therefore, the potential benefit of radioprotection using CeO₂ nanoparticles is of great significance on multiple levels—the most important is its potential impact on human life. This research is relevant to the health and QOL of humans worldwide who are exposed to radiation environments such as those listed below:

- ❖ Patients receiving radiation treatments for cancer;
- ❖ Astronauts in NASA exposed to particle radiation;
- ❖ Military and civilians potentially exposed to radiation in battle, terrorism or occupational exposure.

Verification of the effectiveness of nanoparticles as radioprotectors opens the field for future studies that would examine, in depth, the mechanism, tissue distribution and safety of CeO₂ nanoparticles, prior to utilization in Phase I clinical trials. In the end, these studies may lead to faster recovery and improved QOL for the patients suffering from radiation damage.

Protection of radiation-induced pneumonitis using CeO₂ nanoparticles

Radiotherapy as a treatment for lung cancer

Radiotherapy is an effective treatment option for lung

cancer. However, lung tissue is particularly sensitive to radiation. Thus, the efficacy of radiotherapy is limited by the low tolerance of lung tissue to radiation exposure, and medical professionals seek to optimize the ratio of tumor debulking to lung toxicity. Unfortunately, 30% of patients that receive radiation during their treatment for lung cancer experience clinically significant lung injury (16), and there is no effective therapeutic available for the prevention of acute or chronic radiation-induced pneumonopathy (17). The availability of a radioprotective therapeutic that selectively protects normal lung tissue from radiation-induced-damage would significantly improve the ability of medical professionals to treat patients with lung cancer.

CeO₂ nanoparticles exhibit selective radioprotection of lung fibroblasts in vitro

Normal lung fibroblasts (CCL-135), pre-treated with CeO₂ nanoparticles (10 nM) were exposed to 20 Gy. A Cell Titer-Glo Luminescent Cell Viability Assay (which signals the presence of metabolically active cells) was performed 48 hours after irradiation, and the irradiated normal lung fibroblasts that received CeO₂ nanoparticles pre-treatment had increased viability when compared to irradiated normal cells that did not receive CeO₂ nanoparticles treatment (Figure 3A). When the same experiment was performed on a non-small cell lung cancer cell line (A549), there was no protection (Figure 3B) (15).

In a similar study, normal lung fibroblast (CCL 135) and lung cancer cells (A549) were pretreated with 10 nM

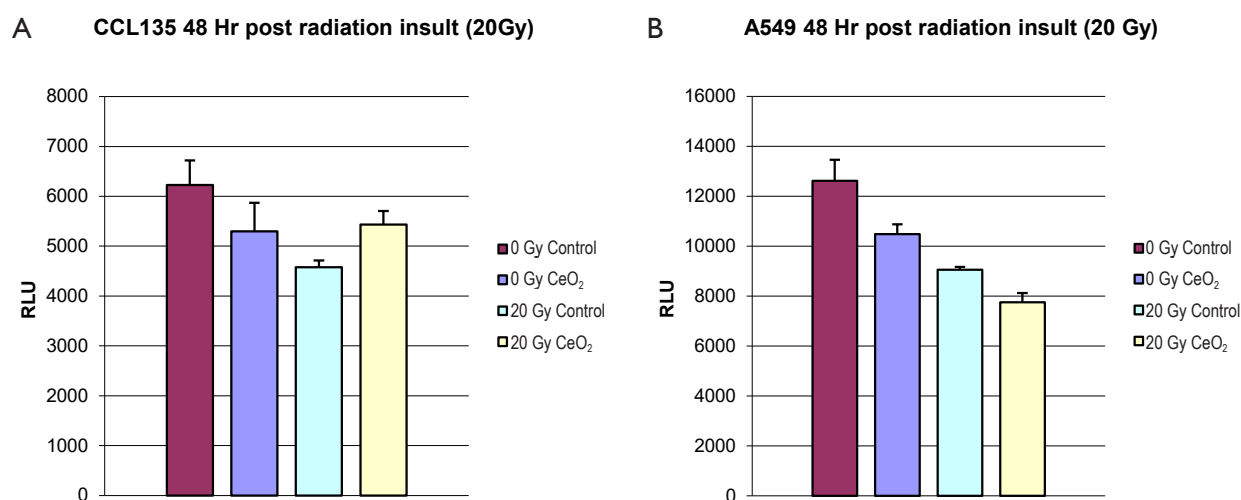


Figure 3 CeO₂ nanoparticles exhibit selective protection of lung fibroblasts. Radiation protection of A. normal lung cells (CCL 135) by CeO₂ nanoparticles; B. No protection observed in lung cancer cells (A549). With permission from Baker C.H. 2009. Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. *Nanomedicine* 5:225-31

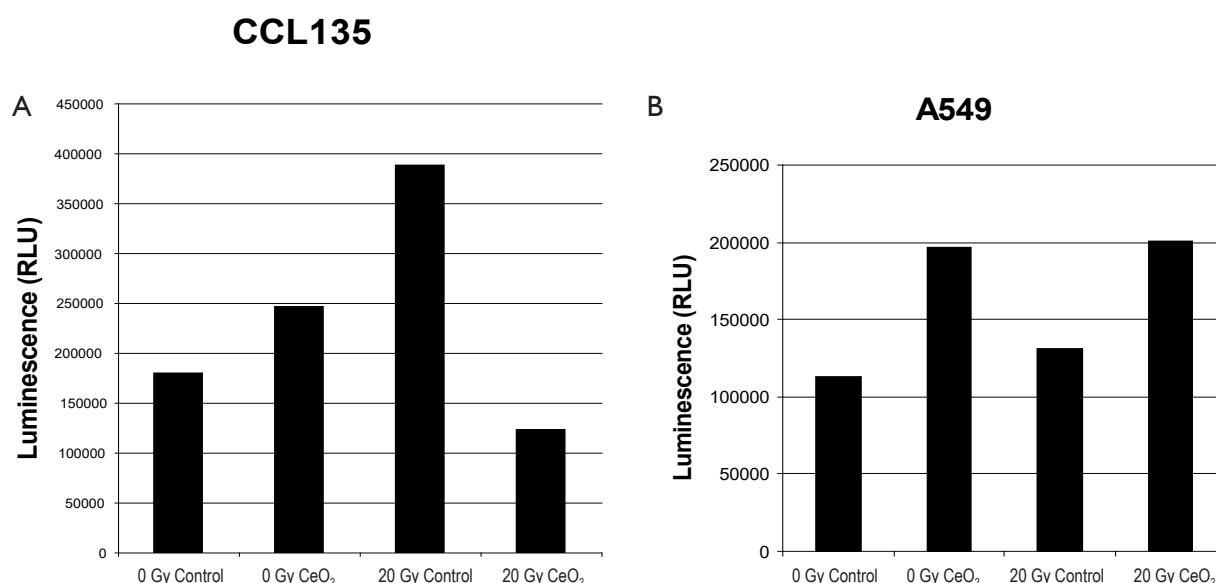


Figure 4 Protection of radiation-induced apoptosis by CeO₂ nanoparticles in normal lung cells. Radiation-induced apoptosis of A. normal lung cells (CCL 135) and B. lung cancer cells (A549). Cells were exposed to 20 Gy radiation in the absence or presence of 10 nM CeO₂ nanoparticles and Caspase 3/7 activity was measured by the Caspase-Glo 3/7 assay. Luminescence is proportional to the amount of caspase activity present. With permission from Baker C.H. 2009. Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. *Nanomedicine* 5:225-31

CeO₂ nanoparticles for 24 hours. Cells were then irradiated with 20 Gy and incubated for 48 hours and assayed for Caspase3/7 activity, which is a protein that is activated during apoptosis. In the presence of CeO₂ nanoparticles, normal cells did not undergo radiation-induced apoptosis

(Figure 4A). In sharp contrast, CeO₂ nanoparticles did not protect the A549 cells from radiation-induced apoptosis (Figure 4B) (15).

Radiation-induced damage and oxidative stress are closely tied. Irradiated cells produce damaging ROS. Previous

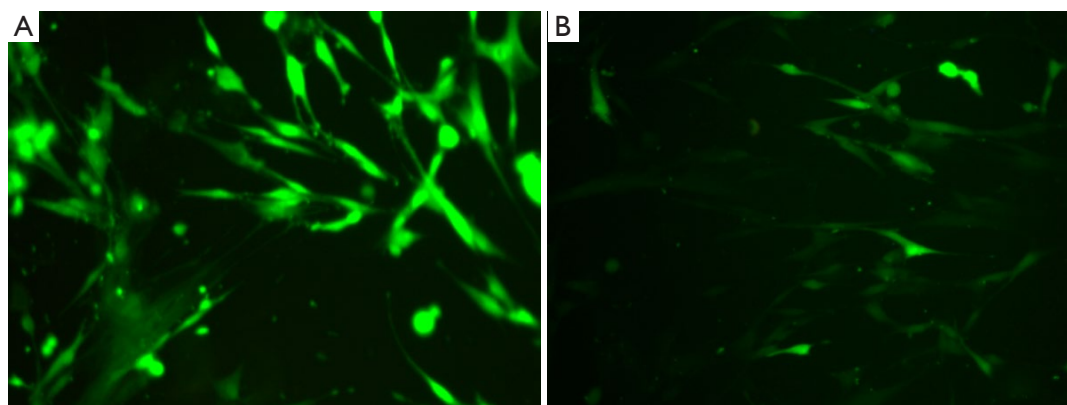


Figure 5 ROS expression in irradiated normal lung fibroblasts. 4 hours post radiation, the levels of ROS were detected in A. irradiated normal lung fibroblasts and B. irradiated normal lung fibroblasts pretreated with CeO₂. *Unpublished data from Cheryl H. Baker*

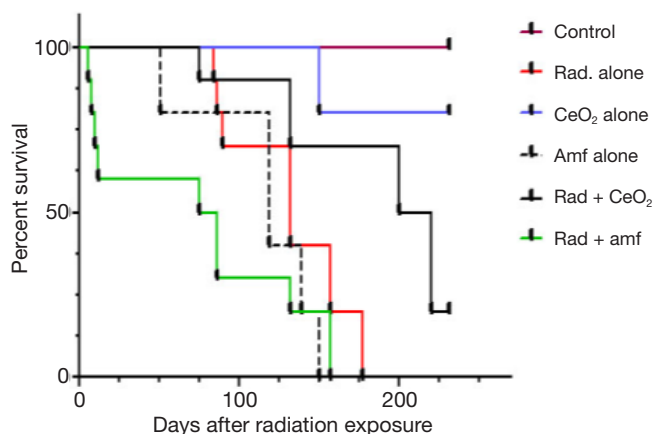


Figure 6 Tolerability of CeO₂ nanoparticles in mice. CeO₂ were well tolerated by mice and the median survival of radiated mice was significantly increased in mice pretreated with 15 nM (0.00001 mg/kg) CeO₂ (50% alive on day 225) as compared to mice treated with radiation alone (50% alive on day 132) or pretreated with 150 mg/kg Amifostine before radiation (50% alive on day 81). Please note that 20% of mice treated with CeO₂ alone were terminated on day 150 for histology analysis. *With permission from Baker C.H. 2009. Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. Nanomedicine 5:225-31*

studies show that CeO₂ nanoparticles exhibits SOD-mimetic activity. To investigate whether CeO₂ nanoparticles can decrease intracellular ROS post irradiation, normal lung fibroblasts were treated with CeO₂ nanoparticles (10 nM) for 24 hours and then irradiated (20 Gy). Intracellular ROS was imaged using the Image-iT Live Green Reactive Oxygen

Species Detection Kit. Control cells were irradiated in the absence of CeO₂ nanoparticles (*Figure 5A*). Results show that CeO₂ nanoparticles decreased the radiation-induced accumulation of ROS (*Figure 5B*). These *in vitro* results show that CeO₂ nanoparticles selectively conferred protection against radiation-induced cell death in normal cells (and not cancer cells) (15).

CeO₂ nanoparticles treatment decreases radiation-induced pneumonitis in murine model

Radiation pneumonitis and subsequent pulmonary fibrosis can significantly decrease the QOL of humans exposed to radiation. In an attempt to administer nanoparticles to live animals and to evaluate the radiation protection activity of CeO₂ nanoparticles, the survival of non-tumor bearing athymic nude mice was measured. Non-tumor bearing athymic nude mice were exposed to fractionated doses of 30 Gy radiation (weekly administration of 5 Gy) in the presence or absence of twice weekly i.p. injections of CeO₂ nanoparticles or i.p. injections of Amifostine 30 minutes prior to radiation. Results show (*Figure 6*) that CeO₂ nanoparticles are well tolerated by athymic nude mice and protect mice from radiation-associated death. All control mice lived until termination date of 231 days. In mice treated with CeO₂ nanoparticles alone, 20% were sacrificed on day 150 for histology analysis. The remaining 80% were alive until the termination date of 231 days. After treatment with radiation alone, Amifostine alone, and a combination of radiation and CeO₂ nanoparticles, or radiation and Amifostine, the median survival time was 132, 119, 225,

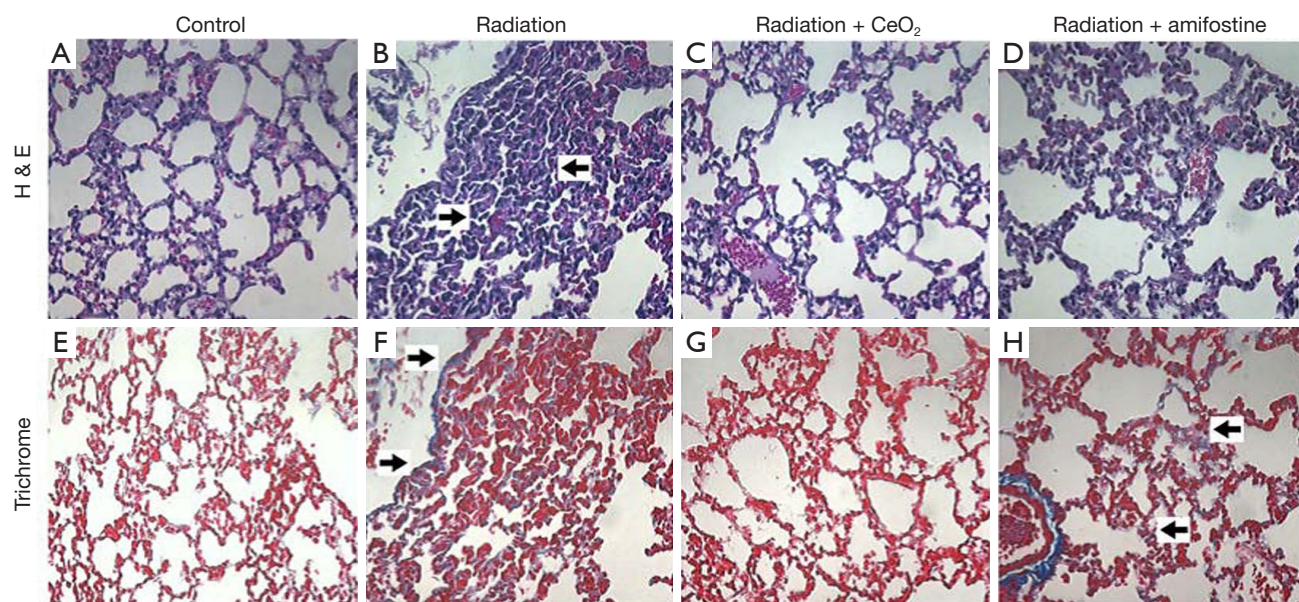


Figure 7 CeO₂ nanoparticles protect lungs from radiation-induced pneumonitis. H&E stains to assess lung damage in normal lungs (A), lungs from mice treated with radiation alone (B), lungs from mice treated with radiation plus CeO₂ (C) and lungs from mice treated with radiation plus Amifostine (D). The H&E stains show significant lung damage in mice treated with radiation (B). Radiation-induced cell damage is protected in lungs of mice treated with radiation in combination with CeO₂ (C) and these lungs appear normal shown in control (A). The amount of fibrosis and collagen deposition (indicative of chronic lung conditions) was measured by using Masson's Trichrome stain. Results show that fibrosis and collagen deposition (indicated by arrows) were common in the lungs of those mice given radiation alone (F) and in lungs of those mice given a pretreatment of Amifostine (H). The amount of fibrosis and collagen deposition in lungs of mice treated with radiation in combination with CeO₂ (G) was minimal and these lungs appeared normal (E). *With permission from Baker C.H. 2009. Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. Nanomedicine 5:225-31*

and 81 days, respectively (control versus radiation, $P < 0.019$; control versus CeO₂, $P < 0.66$; control versus Amifostine, $P < 0.0370$; radiation versus radiation and CeO₂, $P < 0.0041$; radiation versus radiation and Amifostine, $P < 0.0432$). In contrast, Amifostine was highly toxic, as shown by the significant difference in median survival time (as compared to control mice). In summary, these results suggest that CeO₂ nanoparticles are well tolerated by mice and have a significant advantage over the clinically used Amifostine (15).

To determine the degree of radiation-induced pneumonitis, the lungs were harvested and processed for histology and hematoxylin and eosin (H&E) staining. The lungs from mice in the control group (radiation alone) showed visible pneumonitis, with extensive macrophage invasion; whereas the lungs from irradiated mice receiving CeO₂ nanoparticles showed no visible pneumonitis and appeared normal (Figure 7). In addition, the amount of fibrosis and collagen deposition (indicative of chronic lung conditions) was measured in the lungs of control mice (no

radiation/normal lungs), or in lungs of those mice treated with radiation alone, radiation plus CeO₂, or radiation plus Amifostine, using Masson's Trichrome stain. The histology analyses show that fibrosis and collagen deposition were common in the irradiated lungs of those mice given radiation alone and of those mice given a pretreatment of Amifostine (Figure 7). Furthermore, analysis indicated that collagen deposits were relatively recent, due to the faint blue stain, as compared to dark blue staining of older, more cross-linked collagen seen in human chronic lung diseases. In sharp contrast, no significant Trichrome staining was observed in normal lungs (control) or in those irradiated lungs of mice treated with CeO₂ (15).

CeO₂ nanoparticles treatment reduces over-expression of Transforming Growth Factor-beta (TGF- β), a marker for fibrosis

Athymic mice were randomized into two groups. Group

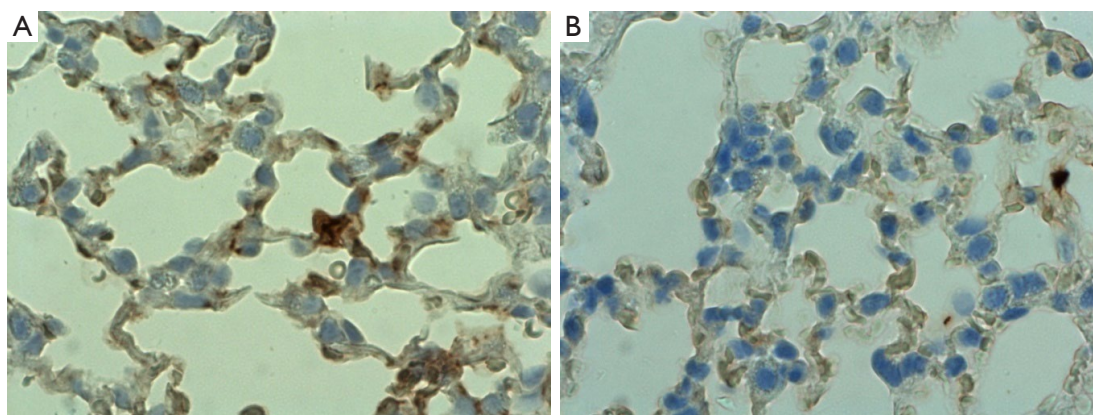


Figure 8 CeO₂ nanoparticles reduces TGF- β expression post radiation. 120 days after XRT (30 Gy) fractionated over 5 doses and 2 weeks mice that received nanoceria treatment had significantly less TGF- β deposition. A. Lung tissue from untreated animal; B. Lung tissue from treated animal (0.005 mg/kg). *Unpublished data from Cheryl H. Baker*

1 received 0.005 mg/kg of CeO₂ nanoparticles prior to irradiation, while group 2 received saline. The mice were irradiated in the ventral thorax with 30 Gy X-rays (fractionated into 5 doses over two weeks). The mice were sacrificed 120 days after irradiation, and the lungs extracted for immunohistochemistry. Slides of lung tissue were stained using a primary antibody (monoclonal mouse anti-mouse TGF- β 1 and secondary antibody (goat anti-mouse HRP), and the slides were counterstained with hematoxylin. The stained slides were imaged with light microscope using oil immersion at 1,000 $\times$ (Figure 8). The images demonstrate a significant level of TGF- β expression in lungs of the untreated animals. Since high levels of TGF- β expression is linked to lung fibrosis and pneumonopathy (17), the decrease in TGF- β expression in the animals that received CeO₂ nanoparticles treatment (as compared to control) indicates that CeO₂ nanoparticles protected the mice from radiation-induced pneumonopathy (15).

Harnessing nanoparticles to improve toxicity after head and neck radiation

Radiation therapy has been a major modality employed in the treatment of head and neck cancer for decades. Unfortunately, the tissues in the head and neck region are exquisitely sensitive to the acute and late effects of radiation treatment (18,19). Due to these toxicities, head and neck cancer patients have a uniquely difficult time during a course of radiation. Many patients will require hospitalization, feeding tube placement, pain medications, and intravenous hydration in order to complete the prescribed course

of treatment. Moreover, these patients often face long-term difficulties with eating, speaking, tasting, dry mouth, decreased range of motion, and wound healing (20). The need to improve toxicity associated with the radiotherapeutic treatment of head and neck cancer is significant.

Recently published American Society of Clinical Oncology (ASCO) guidelines state that Amifostine “may be considered during fractionated radiation therapy (21).” However, these guidelines do not support the use of Amifostine in the use of concurrent chemoradiation, which is presently the standard of care in the treatment of many head and neck cancer patients (21). Moreover, the ability of Amifostine to ameliorate radiation induced dermatitis and mucositis has not been adequately established (21). Hence, there remains a substantial clinical need for a radioprotective agent that can be delivered with relative ease, is long lasting, well-tolerated, and can protect a spectrum of sensitive normal tissues that are responsible for a significant reduction in QOL. In the present report, we show that CeO₂ nanoparticles represent a novel approach to the protection of salivary and skin tissue from radiation-induced damage and report their efficacy as a new radioprotective compound on athymic nude mice receiving radiotherapy to the head and neck.

Effects of CeO₂ nanoparticles on athymic nude mice exposed to radiation to the head and neck region

Sialometry analysis demonstrated a statistically significant difference in salivary flow production between the control group that received 30 Gy/6 fractions of radiation and mice

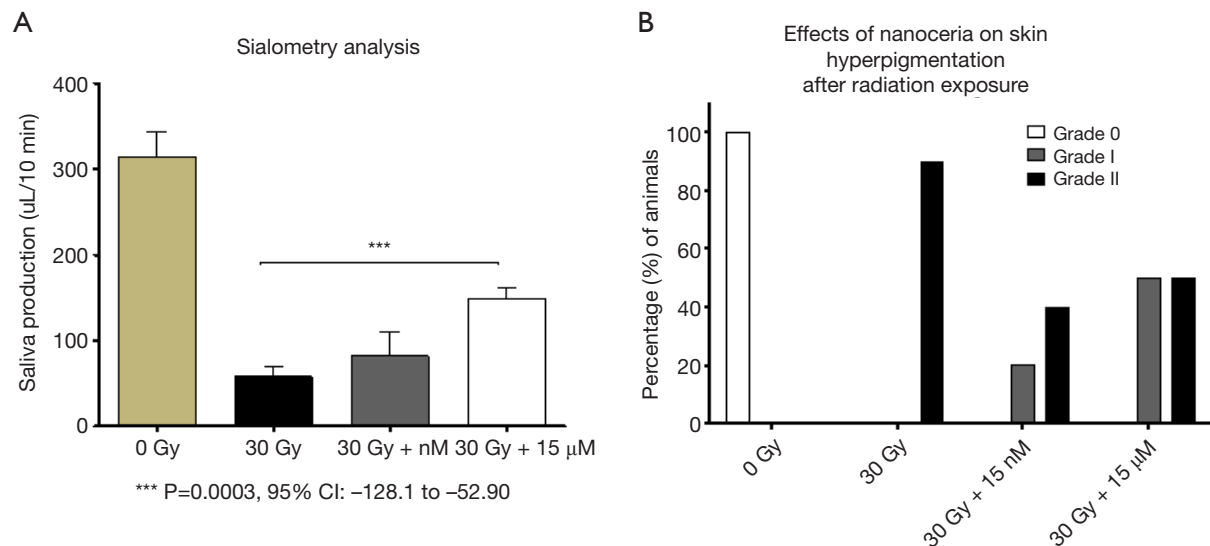


Figure 9 Effects of CeO₂ nanoparticles on athymic nude mice exposed to radiation to the head and neck region. A. Effects of nanoceria on skin hyperpigmentation after radiation exposure using the NCI common terminology criteria for adverse events (CTC 3.0v). Results demonstrated a statistically significant difference in salivary flow production between the control group that received 30 Gy/6 fractions of radiation and mice treated with 30 Gy/6 fractions of radiation that received concomitant treatment with CeO₂ nanoparticles; B. Mice treated with 15 nM CeO₂ nanoparticles resulted in a lower incidence of grade II (33.33%) and a higher incidence of Grade I (66.67%). In sharp contrast, mice treated with 15 μ M CeO₂ nanoparticles had an equal incidence of Grade I and II hyperpigmentation (50% each). *With permission from Baker C.H. 2012. Harnessing nanoparticles to improve toxicity after head and neck radiation. Nanomedicine 8:1223-31*

treated with 30 Gy/6 fractions of radiation that received concomitant treatment with CeO₂ nanoparticles (Figure 9A). The mean stimulated salivary flow rate for the non-radiated group was 313.691 μ L/10 min, while the radiated control group had a mean salivary flow of 115.257 μ L/10 min. Furthermore, the radiated groups that received either low concentration of CeO₂ nanoparticles (15 nM) or high concentration of CeO₂ nanoparticles (15 μ M) had an increase in salivary flow production (mean salivary flow volumes of, 166.825 μ L/10 min and 203.925 μ L/10 min, respectively) when compared to the “no nanoparticle” radiated group 12 weeks after radiation exposure.

While 100% of the skin hyperpigmentation observed in mice treated with radiation alone was recorded as Grade II, mice treated with 15 nM CeO₂ nanoparticles resulted in a lower incidence of grade II (33.33%) and a higher incidence of Grade I (66.67%). In sharp contrast, mice treated with 15 μ M CeO₂ nanoparticles had an equal incidence of Grade I and II hyperpigmentation (50% each) (Figure 9B). Sialometry analysis demonstrated a statistical significant difference in the stimulated salivary flow, between the radiated control group and the group receiving radiation and 15 μ M CeO₂ (P value:

0.0003, 95% CI: -128.0 to -52.90).

An inverse correlation was observed between the incidence of Grade 3 radiation-induced dermatitis and the concentration of CeO₂ nanoparticles given (Figure 10). The incidence of Grade 3 dermatitis 1 week after radiation was decreased in the 15 μ M CeO₂ group compared to the non-CeO₂ controls (10% vs. 100% incidence of Grade 3 dermatitis, respectively). This effect was not appreciated in the 15 nM CeO₂ group. Furthermore, animals exposed to radiation and either 15 nM or 15 μ M concentration of CeO₂ nanoparticles showed swifter resolution of radiation dermatitis when compared to the control “no-nanoparticle” radiated group. For example, complete healing was observed in 60% of animals pre-treated with 15 μ M of CeO₂ nanoparticles before radiation, vs. 10% on the radiated control group, at 12 weeks post-radiation (Figure 10).

Effects of CeO₂ nanoparticles on the apoptotic index of salivary glands parenchymal cells after radiation to the head and neck region

The parotid, sublingual and submandibular glands were

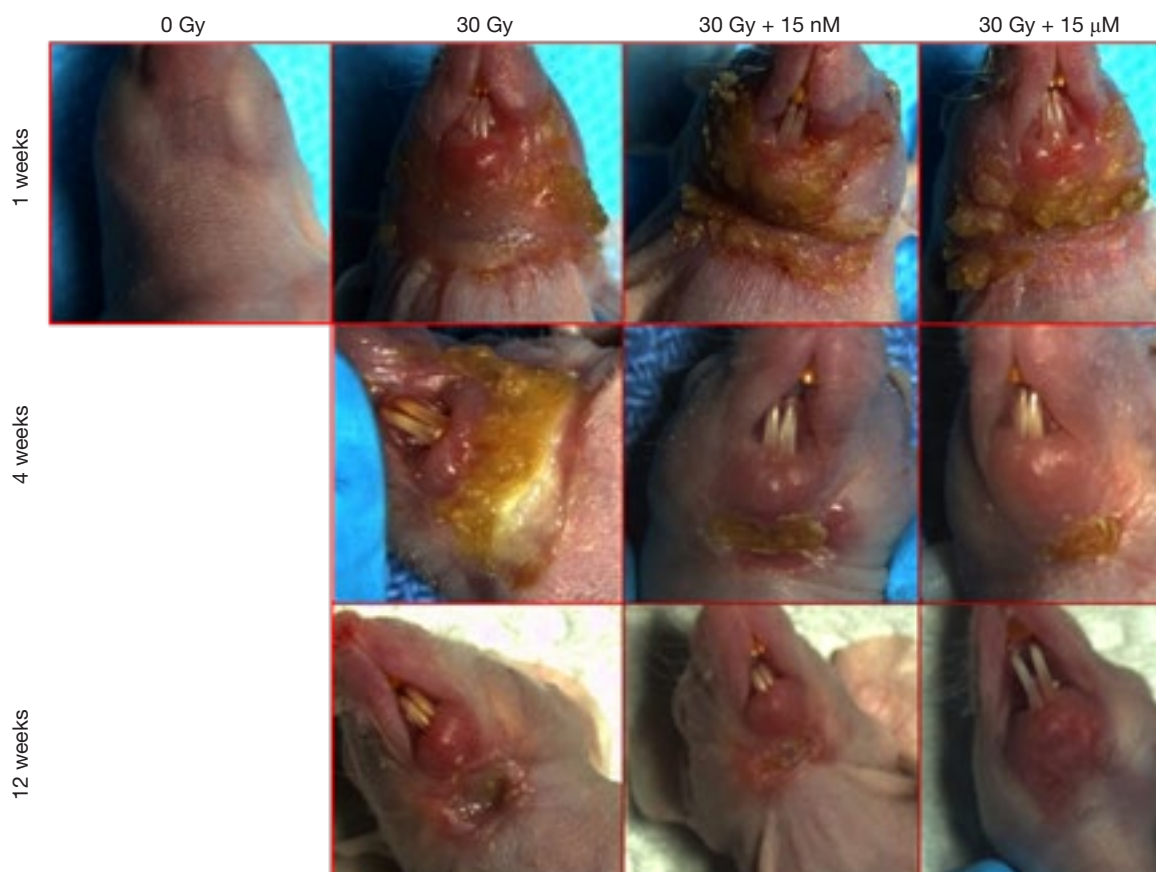


Figure 10 Macroscopic evaluation of radiation-induced dermatitis of athymic mice exposed to 30 Gy in 6 fractions to the head and neck region. An inverse correlation was observed between the incidence of Grade 3 radiation-induced dermatitis and the concentration of CeO₂ nanoparticles given. Animals exposed to radiation combined with either 15 nM or 15 μM concentration of CeO₂ nanoparticles showed swifter resolution of radiation dermatitis when compared to the control “no-nanoparticle” radiated group at 12 weeks post-radiation. *With permission from Baker C.H. 2012. Harnessing nanoparticles to improve toxicity after head and neck radiation. Nanomedicine 8:1223-31*

independently analyzed and the acinar cell apoptotic index was determined using TUNEL analysis. Our results indicate a dose dependent decrease in the apoptotic index for the individual glands after radiation, indicative of the radioprotective nature of the nanoparticles (*Figure 11A*). Complementary analysis of the effects of CeO₂ nanoparticles combined with radiation on all major salivary gland yielded a similar response (*Figure 11B*). The overall apoptotic index baseline of acinar cells for the non-radiated group was 1.43%, while radiation-induced damage increased the apoptotic rate to 19.91%. Meanwhile, after treatment with radiation, both (15 nM and 15 μM) CeO₂ nanoparticle treated groups exhibited an apoptotic index of 8.17% and 4.67%, respectively. Statistical analysis demonstrated a significant difference between the “no-nanoparticle” treated group and the 15 μM CeO₂ treated

group (P value: 0.0270, 95% CI: 2.77 to 27.03). Lastly, a comparison between the group that received a combination of nanoparticles plus radiation and the control group (i.e., “no-nanoparticle” “no-radiation” controls) was performed to quantify the degree of radioprotection from apoptotic death compared to virgin salivary tissue. Comparison of the apoptotic index of the 15 μM CeO₂ nanoparticle group that received radiation versus the “no-radiation” “no-nanoparticle” control group showed no statistical difference (P value: 0.1155, 95% CI: -8.534 to 1.378).

On the other hand, the apoptotic index of the 15 μM CeO₂ nanoparticle treated group that did not receive radiation and the non-radiated “no-nanoparticle” control group showed no statistical difference between them. These results suggest that exposure to CeO₂ nanoparticles does

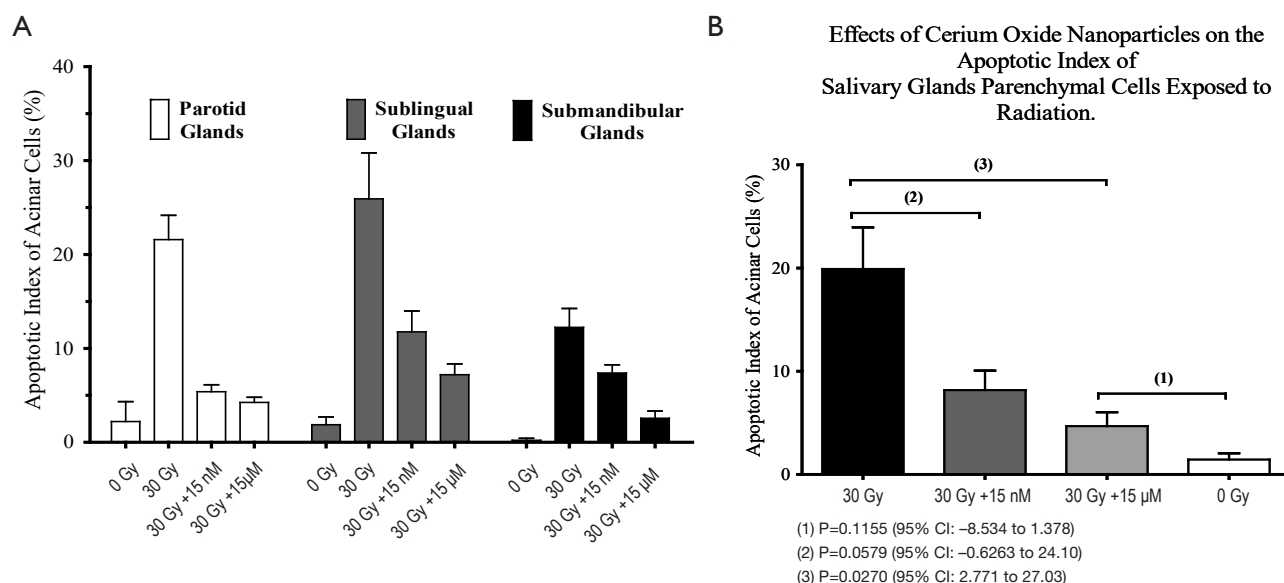


Figure 11 Effects of CeO₂ nanoparticles on the apoptotic index of salivary glands parenchymal cells after radiation to the head and neck region. A. Radiation-induced apoptosis of salivary glands (Parotid, Sublingual and Submandibular) parenchymal cells. Parotid glands of mice showed an increase in apoptotic index after radiation (22%) as compared to non-irradiation (2.2%) and to mice that received either 15 nM or 15 μM CeO₂ nanoparticles (5.32% and 4.25%, respectively). Non-irradiated sublingual glands had a baseline apoptotic index of 1.87%, which increased to 26% after radiation. Pre-treating with either 15 nM or 15 μM CeO₂ nanoparticles resulted in a reduction in the magnitude of elevation to 11.8% and 7.2%, respectively after radiation. Non-irradiated submandibular glands had a baseline apoptotic index of 0.2%. While radiation increased the index to 12.2%, by pre-treating with CeO₂ (15 nM or 15 μM) the magnitude of elevation was decreased to 7.4% and 2.6% respectively; B. Complementary analysis of the effects of CeO₂ nanoparticles combined with radiation on all major salivary gland yielded a similar response to those shown in (A). *With permission from Baker C.H. 2012. Harnessing nanoparticles to improve toxicity after head and neck radiation. Nanomedicine 8:1223-31*

not result in adverse effects to acinar cells.

CeO₂ nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of ROS and upregulation of super oxide dismutase-2

In the context of colorectal carcinomas, damage on surrounding healthy cells which have been inadvertently exposed to ionizing radiation has been exacerbated during radiation treatment since the colon is untethered and mobile, making it particularly susceptible to physical perturbation, such as bladder filling or breathing, which may cause unintended radiation exposure to nearby tissue. Ionizing radiation insult to the tissue causes DNA damage and free radical formation, which leads to stress-induced programmed cell death-apoptosis. In the long term, this damage leads to bowel obstruction, fistula, perforation, or hemorrhage, and these injuries often require further

treatment, in particular, more invasive surgery (22). This study is the first to show that CeO₂ nanoparticles confer radioprotection on colon intestinal cells by exerting free radical scavenger properties and SOD mimetic properties.

CeO₂ nanoparticles reduce ROS levels and protect normal human colon cells from radiation-induced cell death *in vitro*

In order to investigate the effects of CeO₂ nanoparticles on ROS production, normal human colon cells (CRL 1541) were exposed to increasing concentrations of CeO₂ nanoparticles 24 hours prior to a single exposure of 20 Gy radiation. ROS production was measured using the Image-iT LIVETM green ROS detection kit. Results show that when radiation was administered as single therapy, the qualitative production of ROS was significantly increased. However, when CeO₂ nanoparticles were administered 24 hours prior to radiation, the presence of CeO₂ nanoparticles significantly

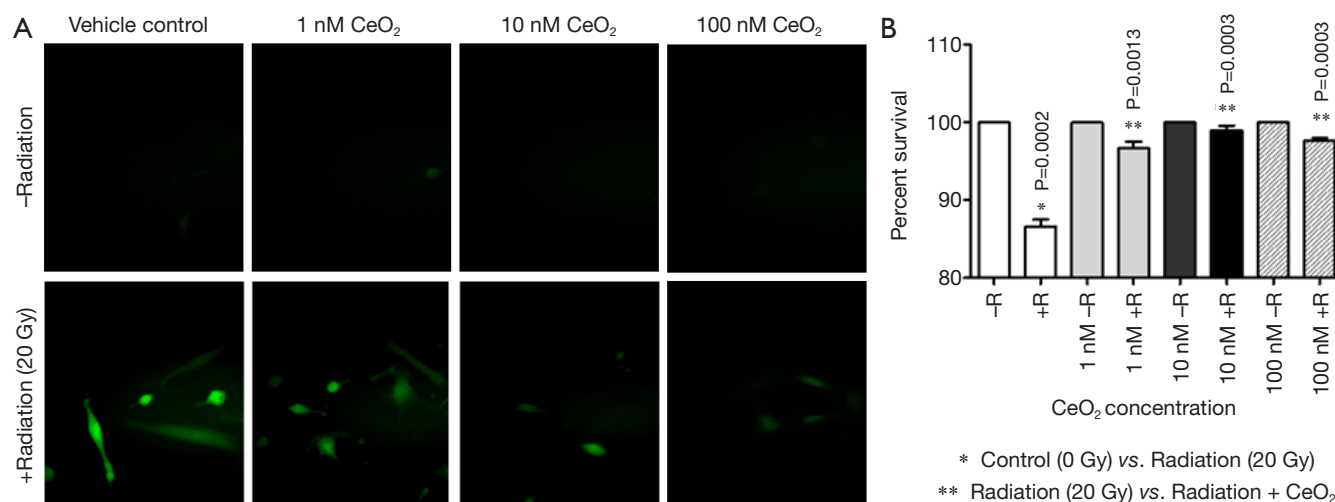


Figure 12 CeO₂ nanoparticles protect normal colon cells against radiation-induced cell damage. CeO₂ nanoparticles protect normal human colon cells against radiation-induced cell damage. A. ROS production of normal human colon cells (CRL 1541) immediately following 20 Gy radiation exposure with pretreatment of 1, 10, or 100 nM CeO₂ nanoparticles was significantly reduced as compared to cells exposed to radiation alone; B. CRL 1541 cells were exposed to 20 Gy radiation in the absence or presence of 1, 10, or 100 nM CeO₂ and 96 hours after exposure cell viability was measured by Cell Titer-Glo Luminescent Cell Viability Assay (cell number correlates with luminescent output (RLU)). *With permission from Baker C.H. 2010. Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. Nanomedicine 6:698-705*

decreased the ROS production, in a dose-dependent manner (Figure 12A). There was no observable difference in ROS production between the control (non-irradiated cells) and the non-irradiated cells treated in combination with increasing concentrations of CeO₂ nanoparticles (Figure 12A) (23).

In another set of experiments, normal human colon cells (CRL 1541) were exposed to increasing concentrations of CeO₂ nanoparticles added 24 hours prior to a single exposure of 20 Gy. Ninety-six hours later, cell viability was measured. Results show that when radiation was administered as single therapy, the number of viable cells in culture was significantly decreased as compared to control (15%). However, when 1, 10 or 100 nM of CeO₂ nanoparticles were administered 24 hours prior to radiation, the CeO₂ nanoparticles significantly protected the cells from radiation-induced cell death (3% for 1 nM, 1% for 10 and 100 nM) (Figure 12B) (23).

CeO₂ nanoparticles induce SOD-2 expression in normal human colon cells in vitro

The effect of CeO₂ nanoparticles (added 24 hrs before radiation) on SOD-2 protein expression on CRL 1541 cells growing in normal growth media was measured. Western

blot analysis show increased levels of SOD-2 in normal colon cells in the presence of CeO₂ nanoparticles and in a dose-dependent fashion, the band intensity of SOD-2 in 100 nM CeO₂ nanoparticles treated cells was roughly 2-fold higher than non-treated control cells. The cells exhibited increased SOD-2 expression with the addition of increasing concentrations of CeO₂ nanoparticles (Figure 13) suggesting that CeO₂ nanoparticles increased normal colon cell SOD-2 expression when added 24 hrs before radiation, conferring cytoprotection from the radiation insult. This phenomenon is corroborated by a corresponding increase in cell survival rates when normal colon cells are treated with increasing doses of CeO₂ nanoparticles (23).

CeO₂ nanoparticles reduce apoptotic cell death in gastrointestinal mice cells in vivo

In an attempt to investigate the ability of CeO₂ nanoparticles to protect the gastrointestinal epithelium of mice against radiation-induced damage, mice were randomized and colon tissues were harvested and processed four hours post radiation. The colonic crypt cells from mice treated with CeO₂ nanoparticles in combination with radiation exhibited a significant decrease in apoptotic colon cryptic

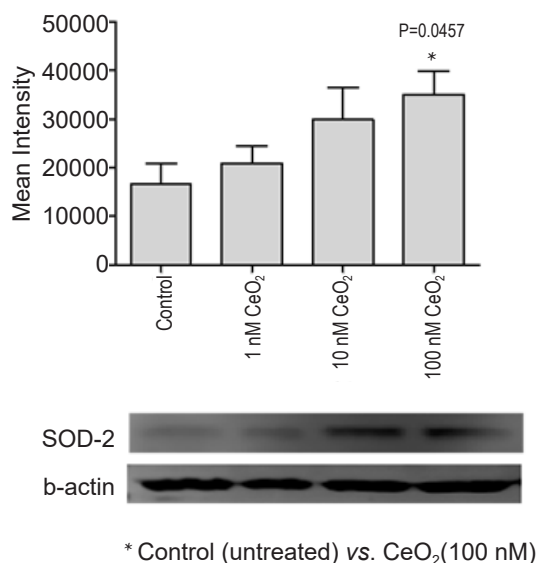
Protein expression of SOD-2 24 hrs post CeO₂ treatment on normal colon CRL 1541 cells

Figure 13 CeO₂ nanoparticles induce protein SOD-2 expression. The effect of CeO₂ nanoparticles on SOD-2 protein expression on CRL 1541 cells growing in normal growth media. The cells exhibited a dose-dependent increase in protein expression of SOD-2 with the addition of increasing concentrations of CeO₂ nanoparticles. The protein band intensity of SOD-2 in cells incubated with 100 nM CeO₂ nanoparticles was roughly 2-fold higher than cells incubated in media alone. *With permission from Baker C.H. 2010. Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. Nanomedicine 6:698-705*

cells (as measured by TUNEL) and Caspase-3 expression as compared to the colonic crypt cells from irradiated (no CeO₂) mice (Figure 14). The number of TUNEL and Caspase-3 positive cells in each colonic crypt decreased by 50% in mice treated with a combination of CeO₂ nanoparticles and radiation, as compared to mice treated with radiation alone. It is interesting to note the decrease in Caspase-3 in mice treated with CeO₂ nanoparticles as compared to control (normal) mice which could be explained by the fact that CeO₂ may reduce the normal intrinsic cell death pathway and/or normal metabolic ROS, as reviewed by Rzigalinski (7).

To demonstrate the ability of the CeO₂ nanoparticles to induce the overexpression of SOD-2 colons from mice were sectioned 24 hours after a single injection of CeO₂

nanoparticles and 10 random crypts per mouse from five different mice per group were stained for SOD-2 expression (Figure 15A). The colonic crypt cells from mice treated with CeO₂ nanoparticles exhibited a 40% increase in SOD-2 expression as compared to untreated (normal) mice (Figure 15B). Immunohistochemical analysis of normal colon from mice treated with CeO₂ nanoparticles show an increase in SOD-2 expression (23).

Discussion

The field of radiation oncology has worked diligently over the last decade to improve radiation delivery techniques in order to spare sensitive structures from the effects of ionizing radiation. These techniques have resulted in improved functional outcomes compared to prior, more rudimentary, radiation techniques. However, the need to attain adequate tumor coverage and the exquisite radiosensitivity of certain normal structures are intrinsic limitations to the magnitude of function and QOL that can be preserved with these techniques. Hence, even with the implementation of these techniques many patients still experience significant acute and late toxicity after radiation treatment that adversely impacts their QOL.

To further improve radiation-induced toxicities we must continue to develop strategies to protect normal tissues from radiation-induced damage. One such strategy is the development of radiation protectors. Several compounds have been described, but Amifostine remains the only agent currently in clinical use (24). Major limitations to the clinical use of Amifostine are its short half-life, daily dosing requirements, toxicity based on route of administration, and its cost (4,6,15,24). Hence, there remains a substantial clinical need for a radioprotective agent that can be delivered with relative ease, is long lasting, well-tolerated, and can protect a spectrum of sensitive normal tissues that are responsible for a significant reduction in QOL.

The above report lends a great deal of credence to the argument for the use of CeO₂ nanoparticles in a therapeutic setting as a free radical scavenger, especially in the context of therapeutic ionizing radiation. As mentioned above, CeO₂ nanoparticles, due to their large surface energy derived from a high surface area to volume ratio and unique valence state oscillations, contain many oxygen vacancies which allow them to be much more efficient than endogenous antioxidants, and to be regenerative in their enzymatic activity, which we hypothesize to be due to the valence reversing from +3 to +4 valence states. Additionally,

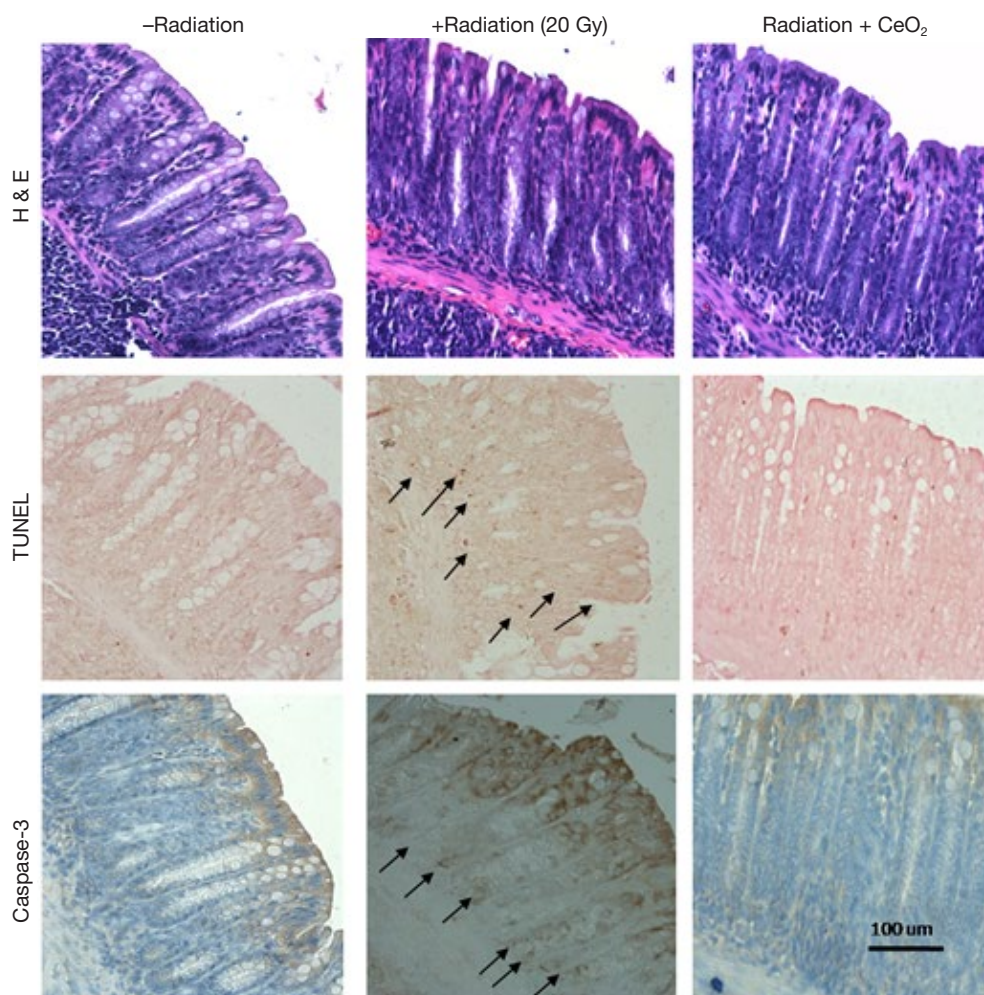


Figure 14 CeO₂ nanoparticles protect normal human colon tissue from radiation-induced cell death. Hematoxylin and Eosin (H&E) stains of murine colons 4 hours post a single dose of 20 Gy radiation. Radiation was administered to the bowel of non-tumor bearing athymic nude mice pretreated with four i.p. treatments of CeO₂ nanoparticles. Results show a significant decrease in apoptotic colon cryptic cells (as measured by TUNEL) and Caspase-3 expression as compared to the colonic crypt cells from mice treated with radiation alone. *With permission from Baker C.H. 2010. Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. Nanomedicine 6:698-705*

mice administered with CeO₂ nanoparticles experience no serious side-effects, demonstrating the low toxicity of CeO₂ nanoparticles (15).

Elevated ROS levels have long been implicated in numerous diseases such as kidney fibrosis (25), chronic inflammation and organ dysfunction, especially when induced by ionizing radiation (26). It is now widely accepted that ROS can interfere in intracellular processes which cause the above mentioned injuries. Thus, the therapeutic value of CeO₂ nanoparticles may be due to their free radical

scavenging properties. Furthermore, CeO₂ nanoparticles as scavenging enzymes, are many times more efficient than SOD, which may be due to the large surface area to volume ratio, as well as the ratio of Ce³⁺/Ce⁴⁺ (7). The *in vivo* experiments also reinforce the conclusion that CeO₂ nanoparticles confer significant protection from ionizing radiation as evidenced by TUNEL and Caspase-3 stains, indicators of cell apoptosis (27).

In the end, while CeO₂ nanoparticles may affect intracellular oxidative pathways, we show clearly that they

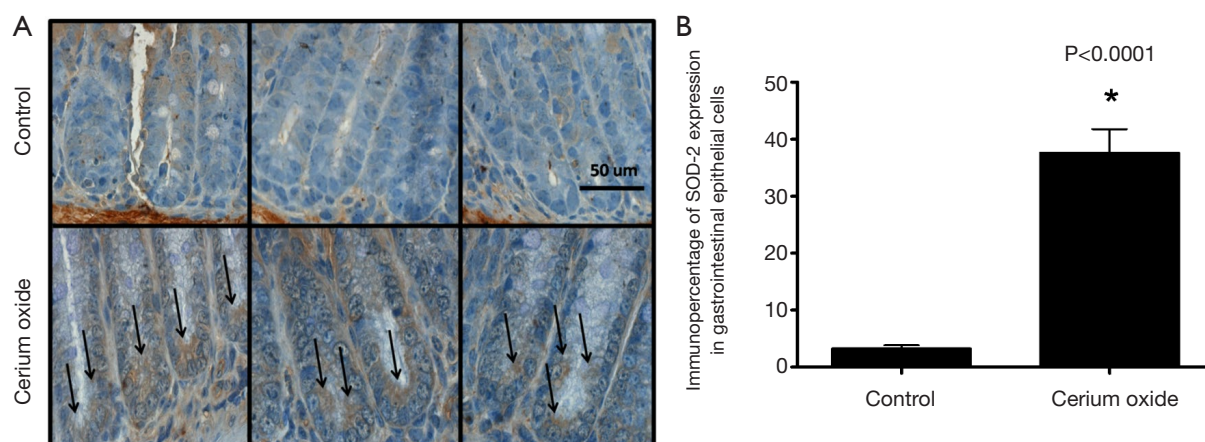


Figure 15 CeO₂ nanoparticles induce SOD-2 expression in normal colon. A. Representative sections of SOD-2 expression (brown staining) in colonic crypts in mice treated with CeO₂ nanoparticles or in normal (control) mice. Colons were collected 24 hours post a single injection of CeO₂ nanoparticles; B. The immunopositive percentage of SOD-2 expression increased by 40% in mice treated with CeO₂ nanoparticles as compared to control mice. Each data point represents the mean $\pm$ SEM from analyzing 10 random crypts per mouse from five different mice which has been expressed as percentage of crypt cells staining positive for SOD-2. *With permission from Baker C.H. 2010. Cerium oxide nanoparticles protect gastrointestinal epithelium from radiation-induced damage by reduction of reactive oxygen species and upregulation of superoxide dismutase 2. Nanomedicine 6:698-705*

are not detrimental; and suspect that the elevated expression of SOD-2 contributes to an increased protection of normal cells against ROS. It is important to note the therapeutic value of free radical scavengers extends beyond protecting against radiation-induced damage to DNA, but also to the reduction in inflammation, fibrosis and organ dysfunction. Thus, we believe that CeO₂ nanoparticles are at the forefront of the effort to utilize emerging nanotechnology to improve QOL and healthcare, and that they hold great potential for future clinical trials.

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Footnote

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