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Review Article

Radiation Protection with Nanoparticles

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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 metallothionine, 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 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 cerium oxide 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 cerium oxide 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.

ABBREVIATIONS

ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; CeO_2: Cerium Oxide; ATP: Adenosine Triphosphate; H&E: Hematoxylin and Eosin; TGF-β: Transforming Growth Factor-beta.

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 metallothionine, 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 (CeO_2) nanoparticles.

Most recently, CeO_2 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 CeO_2 nanoparticles supports a potential role as a biological free radical scavenger or antioxidant. It was suggested that the unique structure of CeO_2 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, CeO_2 nanoparticles are shown to confer protection against radiation-induced cell damage *in* vitro and *in vivo*, suggesting that CeO_2 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.

CeO_2 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 CeO_2 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.

As cellular levels of ROS are tightly controlled in normal, healthy cells [9], the ability to modulate the redox status of cells has applications in diseases where ROS levels have become de-regulated or are altered by treatment. Though more recently linked to cell proliferation and survival, ROS accumulation is generally associated with undesired effects, having been linked to neurodegenerative diseases, diabetes, atherosclerosis, and even aging [9]. With regards to cancer, which causes over 500,000 deaths per year [10], ROS can drive both the initial development and progression, as well as down regulate antioxidant enzymes that normally combat radical production [11]. Studies have shown that CeO_2 nanoparticles possess innate cytotoxicity to cancer cells, anti-invasive properties, and the ability to sensitize cancer cells to radiation induced cell death, while protecting the surrounding normal tissues. Additionally, CeO_2 nanoparticles treatment has been shown to prevent macular degeneration [12] and the formation of neovascular lesions in the retina [13], as well as decrease hepatic ROS levels linked to the progression of diabetes [14]. Thus, CeO_2 nanoparticles have extensive potential as a therapeutic agent for the treatment of a multitude of diseases in which ROS have been implicated.

Cellular Uptake, Biodistribution and Toxicity of CeO_2 Nanoparticles

CeO_2 nanoparticles have been shown to enter mammalian cells in both normal and diseased states [15-17], with significant uptake occurring within 3 hours of exposure in culture [18]. Particle size and surface charge appear to be determinants of CeO_2 nanoparticles uptake and cellular localization [19]. As the differential pH of various sub-cellular localizations has been shown to be a determinant of CeO_2 nanoparticles' anti- or pro-oxidant activity [19], manipulation of CeO_2 nanoparticles to target specific cells or sub-cellular locations is a path that has yet to be fully elucidated and exploited. Several reports have shown CeO_2 nanoparticles (<10 nm) to be well tolerated by animals without inducting obvious toxicity or an immune response across a range of doses [20-23]. When administered intravenously (i.v.) or intraperitoneally (i.p.), studies show that CeO_2 nanoparticles accumulate primarily in the spleen and liver, to a lesser extent in the lungs and kidneys, but not in the heart or brain [22,23]. Tissues such as the breasts and pancreas have not been analyzed for retention, yet nearly half of the injected CeO_2 nanoparticles remained in undetermined locations within the body [23]. Further, CeO_2 nanoparticles were not readily cleared, persisting in the animals for at least 30 days without any appreciable CeO_2 nanoparticles concentration in the urine or feces [22,23], suggesting that other CeO_2 nanoparticles destinations within the body have yet to be identified.

While there are some concerns about the toxicity of nanoparticles, there are very few reports regarding the biologically detrimental effects of CeO_2 nanoparticles. In an article published recently in Toxicology, Park et al. conclude that CeO_2 nanoparticles (15-45 nm; 5-40 μg/ml) induced oxidative stress and cell death in cultured human lung epithelial cells [24]. 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 CeO_2 nanoparticles doses ~1000 times the effective radioprotective dose was recently published [6].

Despite the apparent lack of toxicity in animal models, reports provide conflicting data about the toxicity of CeO_2 nanoparticles in vitro, likely attributable to the impact of undetermined cellular and environmental factors on the manifestation of anti- or pro-oxidant behavior. CeO_2 nanoparticles are toxic to bronchial epithelial lung fibroblasts in culture [24] but non-toxic to mammary epithelial cells [6], macrophages [25], immortalized keratinocytes [22], or immortalized pancreatic epithelial cells [26]. In normal cells to which they are not toxic, the physiological pH is an environment which enables canonical radical scavenging by CeO_2 nanoparticles. Therefore, CeO_2 nanoparticles introduced prior to ROS insult confer protection from the effects of oxidative stress in vitro and in vivo [5,13,27].

Need for a 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 [28]. 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, CeO_2 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 superoxide dismutase (SOD) mimetic activity of CeO_2 [29]. Additionally, the free radical scavenging property of CeO_2 nanoparticles is regenerative[6] which is not the case for other antioxidants. It is believed that due to the chemical nature of CeO_2 nanoparticles, there is an auto-regenerative reaction cycle ($Ce^{3+} \rightarrow Ce^{4+} \rightarrow Ce^{3+}$) continuing on the surface of ceria nanoparticles and is thought to be the current mechanism by which it provides the material with an unprecedented free radical scavenging ability (Figure 1A,B).

CeO_2 Nanoparticles Exhibit in vitro Free Radical Scavenging Ability

The chemistry of engineered CeO_2 nanoparticles supports their potential role as free radical scavengers, antioxidants, in biological systems [28]. It was suggested that the unique surface chemistry of CeO_2 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 a CeO_2 nanoparticle enter mammalian cells [8], decreases the accumulation of ROS, and prevents the activation of the ROS-induced apoptosis [5]. Since cells produce ROS after being exposed to radiation [30], the antioxidant capability of CeO_2 nanoparticles has been suggested as the key mechanism by which CeO_2 nanoparticles confers radioprotection [6]. Furthermore, a study concluded that CeO_2 nanoparticles exhibited superoxide dismutase-mimetic activity [30]. Results supporting the antioxidant properties of CeO_2 nanoparticles is mounting, and many studies suggest that these nanoparticles act as free radical scavengers [6,7,31] and may render protection against chemical insults that promote the production of free radicals [32]. Thus, it has been proposed that CeO_2 nanoparticles may confer radioprotection by scavenging the free radical produced during radiotherapy [6].

CeO_2 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 CeO_2 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 CeO_2 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 CeO_2 nanoparticles-treated animals had little skin damage (Figure 2B). These results suggest that CeO_2 nanoparticles is able to protect mice from a single dose of radiation, and support CeO_2 nanoparticles's role as a radioprotectant [20].

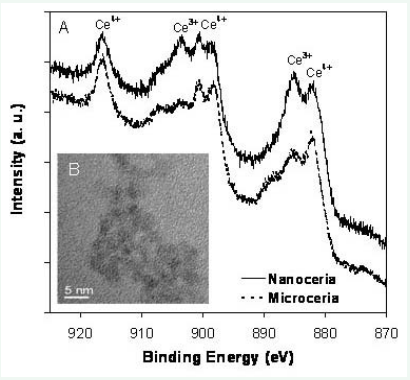

Figure 1 **Characterization of CeO_2 nanoparticles.**

A. X-ray photoelectron spectroscopy (XPS) spectra indicates high concentration of Ce^{3+} in CeO_2 compared to microceria particles. Peaks at 882.1 and 886 eV correspond to Ce^{+4} and Ce^{+3} peaks. Peaks at 918 eV correspond to satellite peaks indicating the presence of Ce^{+4} 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. 5:225-231.*

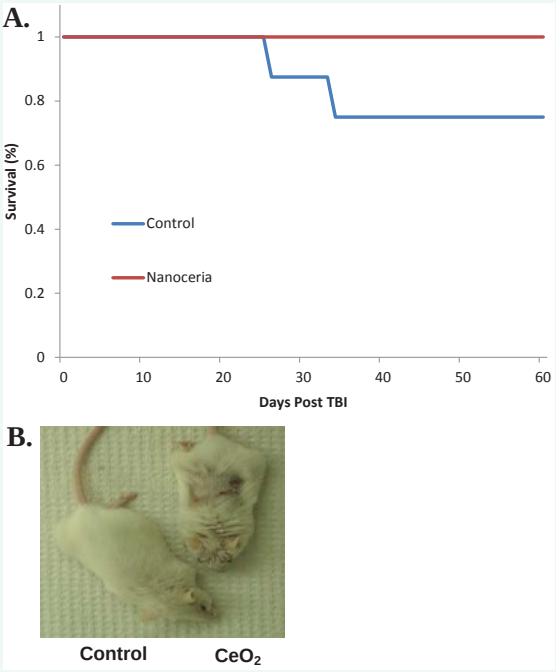

Figure 2 **CeO_2 Nanoparticles Protect Mice from Total Body Irradiation.**

CeO_2 Protects Mice from Total Body Irradiation. A. Survival Curve. B. Mice treated with CeO_2 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.*

CeO_2 Nanoparticles is Well-Tolerated in Athymic Mice

To investigate the acute toxicity of CeO_2 nanoparticles, athymic nude mice were randomized into five groups. Each group received a total nanoparticle dose in the range of 0 (saline), 0.135 mg/kg. 1.35 mg/kg, 13.5 mg/kg, 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 CeO_2 nanoparticles are well-tolerated in mice up to 3 million times the effective dose. Therefore, it was suggested that CeO_2 nanoparticles causes limited toxicity and side effects in mice [20].

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 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 CeO_2 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 quality of life 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 CeO_2 nanoparticles, prior to utilization in Phase I clinical trials. In the end, these studies may lead to faster recovery and improved quality of life for the patients suffering from radiation damage.

Protection of Radiation-Induced Pneumonitis Using CeO_2 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 [33], and there is no effective therapeutic available for the prevention of acute or chronic radiation-induced pneumonopathy [34]. 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.

CeO_2 Nanoparticles Exhibit Selective Radioprotection of Lung Fibroblasts *in vitro*: Normal lung fibroblasts (CCL-135), pre-treated with CeO_2 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 CeO_2 nanoparticles pre-treatment had increased viability when compared to irradiated normal cells that did not receive CeO_2 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) [20].

In a similar study, normal lung fibroblast (CCL 135) and lung cancer cells (A549) were pretreated with 10 nM CeO_2 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 CeO_2 nanoparticles, normal cells did not undergo radiation-induced apoptosis (Figure 4A). In sharp contrast, CeO_2 nanoparticles did not protect the A549 cells from radiation-induced apoptosis (Figure 4B) [20].

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

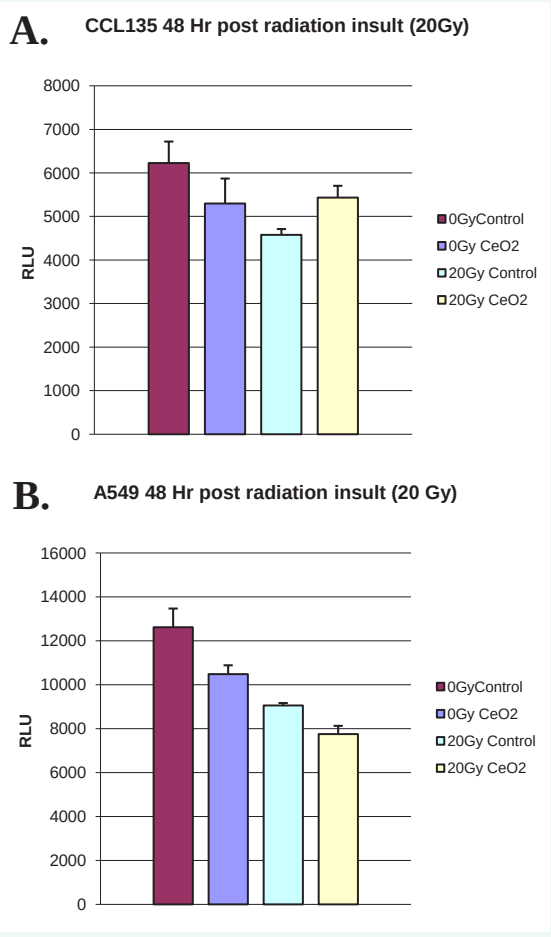

Figure 3 CeO_2 Nanoparticles Exhibit Selective Protection of Lung Fibroblasts.

Radiation protection of A.) normal lung cells (CCL 135) by CeO_2 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. 5:225-231.*

show that CeO_2 nanoparticles exhibits SOD-mimetic activity. To investigate whether CeO_2 nanoparticles can decrease intracellular ROS post irradiation, normal lung fibroblasts were treated with CeO_2 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 CeO_2 nanoparticles (Figure 5A). Results show that CeO_2 nanoparticles decreased the radiation-induced accumulation of ROS (Figure 5B). These *in vitro* results show that CeO_2 nanoparticles selectively conferred protection against radiation-induced cell death in normal cells (and not cancer cells) [20].

CeO_2 nanoparticles Treatment Decreases Radiation-Induced Pneumonitis in Murine Model: Radiation pneumonitis and subsequent pulmonary fibrosis can significantly decrease the quality of life of humans exposed to radiation. In an attempt to administer nanoparticles to live animals and to evaluate the radiation protection activity of CeO_2 nanoparticles, the survival of non-tumor bearing athymic nude mice was measured. Non-tumor bearing athymic nude mice were exposed to fractionated doses of

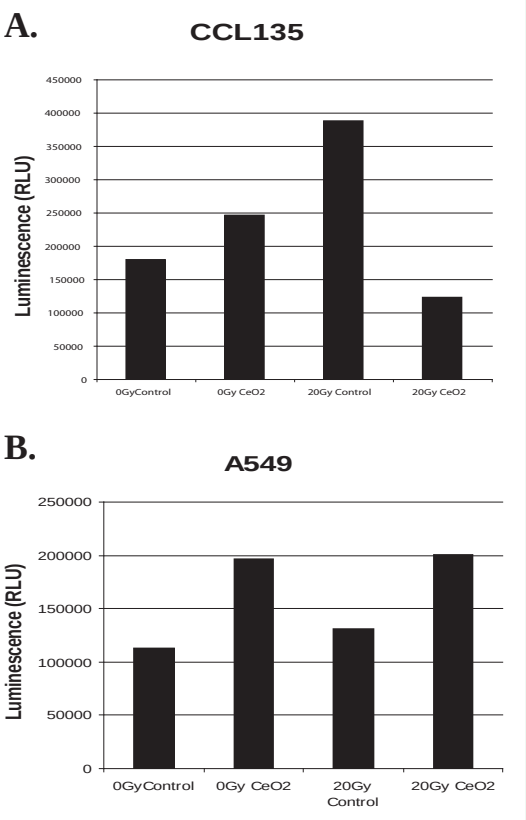

Figure 4 **Protection of Radiation-Induced Apoptosis by CeO_2 Nanoparticles in Normal Lung Cells.**

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 CeO_2 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. 5:225-231.*

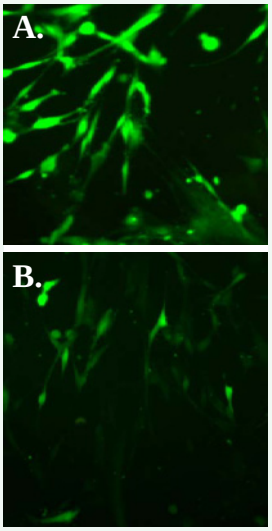

Figure 5 **ROS Expression in Irradiated Normal Lung Fibroblasts.**

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 CeO_2. *Unpublished data from Cheryl H. Baker.*

30 Gy radiation (weekly administration of 5Gy) in the presence or absence of twice weekly i.p. injections of CeO_2 nanoparticles or i.p. injections of Amifostine 30 minutes prior to radiation. Results show (Figure 6) that CeO_2 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 CeO_2 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 CeO_2 nanoparticles, or radiation and Amifostine, the median survival time was 132, 119, 225, and 81 days, respectively (control versus radiation, $P < 0.019$; control versus CeO_2, $P < 0.66$; control versus Amifostine, $P < 0.0370$; radiation versus radiation and CeO_2, $P < 0.0041$; 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 CeO_2 nanoparticles are well tolerated by mice and have a significant advantage over the clinically used Amifostine [20].

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 CeO_2 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 CeO_2, 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

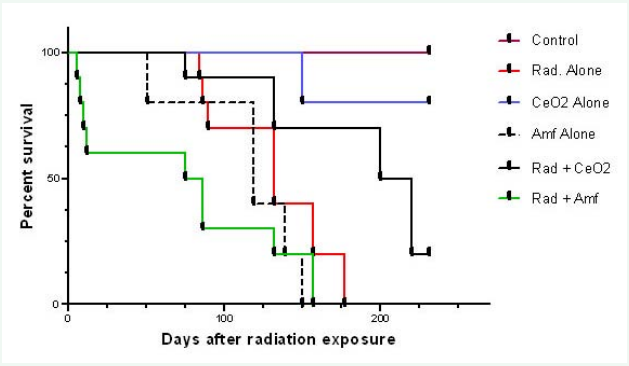

Figure 6 **Tolerability of CeO_2 Nanoparticles in Mice.**

CeO_2 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) CeO_2 (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 CeO_2 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. 5:225-231.*

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 CeO_2 [20].

CeO_2 Nanoparticles Treatment Reduces Over-expression of TGF-β, a Marker for Fibrosis: Athymic mice were randomized into two groups. Group 1 received 0.005 mg/kg of CeO_2 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-β1 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 1000x (Figure 8A,B). 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 [34], the decrease in TGF-β expression in the animals that received CeO_2 nanoparticles treatment (as compared to control) indicates that CeO_2 nanoparticles protected the mice from radiation-induced pneumonopathy [20].

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 [35,36]. 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 [37]. 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 [38]."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 [38]. Moreover, the ability of Amifostine to ameliorate radiation induced dermatitis and mucositis has not been adequately established [38]. Hence, there remains a substantial clinical need

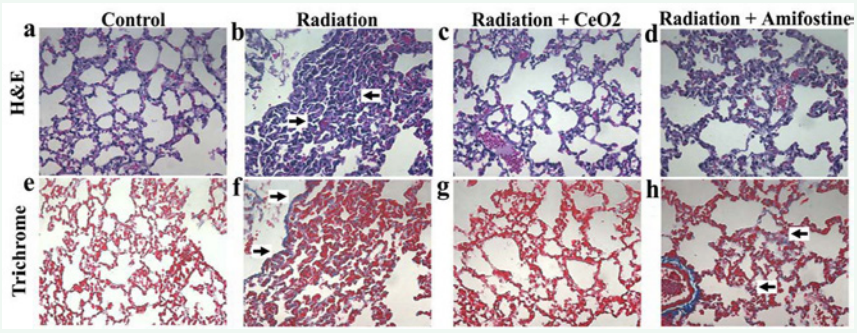

Figure 7 **CeO_2 Nanoparticles Protect Lungs from Radiation-Induced Pneumonitis.**

CeO_2 nanoparticles protect lungs from radiation-induced pneumonitis. Hematoxylin and Eosin (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 CeO_2 (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 CeO_2 (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 CeO_2 (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. 5:225-231.*

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 quality of life. In the present report, we show that CeO_2 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 Cerium Oxide 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 treated with 30 Gy/6 fractions of radiation that received concomitant treatment with CeO_2 nanoparticles (Figure 9A). The mean stimulated salivary flow rate for the non-radiated group was 313.691 µL/10min, while the radiated control group had a mean salivary flow of 115.257 µL/10min. Furthermore, the radiated groups that received either low concentration of CeO_2 nanoparticles (15 nM) or high concentration of CeO_2 nanoparticles (15 µM) had an increase in salivary flow production (mean salivary flow volumes of, 166.825 µL/10min and 203.925 µL/10min, respectively) when compared to the "no nanoparticle" radiated group 12 weeks after radiation exposure [39].

While 100% of the skin hyperpigmentation observed in mice treated with radiation alone was recorded as Grade II, mice treated with 15 nM CeO_2 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 µM CeO_2 nanoparticles had an equal incidence of Grade I and II hyperpigmentation (50% each) (Figure 9B). Sialometry analysis demonstrated a statistical

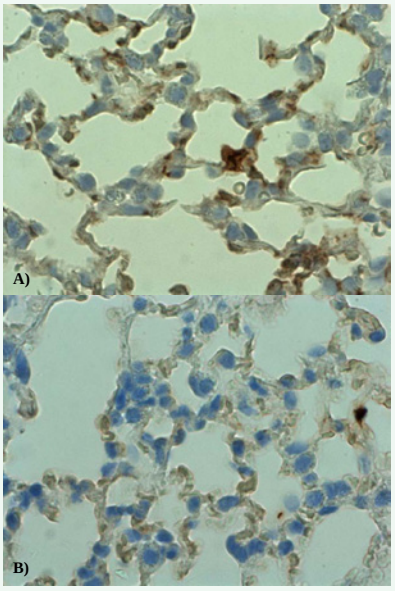

Figure 8 CeO_2 Nanoparticles Reduces TGF-β Expression Post Radiation.

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.*

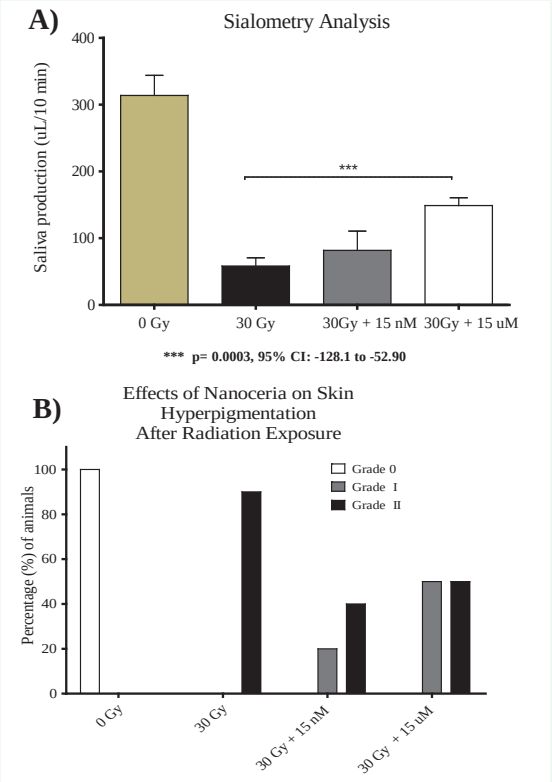

Figure 9 Effects of Cerium Oxide Nanoparticles on Athymic Nude Mice Exposed to Radiation to the Head and Neck Region.

Effects of Cerium Oxide 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 CeO_2 nanoparticles. (B) Mice treated with 15 nM CeO_2 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 µM CeO_2 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. 7:1223-1231.*

significant difference in the stimulated salivary flow, between the radiated control group and the group receiving radiation and 15 µM CeO_2 (P value: 0.0003, 95% CI: -128.0 to -52.90) [39].

An inverse correlation was observed between the incidence of Grade 3 radiation-induced dermatitis and the concentration of CeO_2 nanoparticles given (Figure 10). The incidence of Grade 3 dermatitis 1 week after radiation was decreased in the 15 µM CeO_2 group compared to the non-CeO_2 controls (10% vs. 100% incidence of Grade 3 dermatitis, respectively). This effect was not appreciated in the 15 nM CeO_2 group. Furthermore, animals exposed to radiation and either 15 nM or 15 µM concentration of CeO_2 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 µM of CeO_2 nanoparticles before radiation, vs 10% on the radiated control group, at 12 weeks post-radiation (Figure 10) [39].

Effects of Cerium Oxide 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 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 CeO_2 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) CeO_2 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 CeO_2 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 CeO_2 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) [39].

On the other hand, the apoptotic index of the 15 µM CeO_2 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 CeO_2 nanoparticles does not result in adverse effects to acinar cells [39].

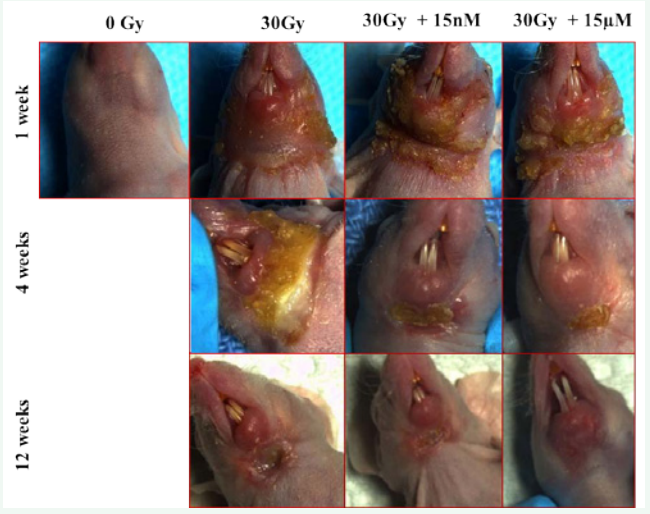

Figure 10 **Macroscopic Evaluation of Radiation-Induced Dermatitis of Athymic Mice Exposed to 30 Gy in 6 Fractions to the Head and Neck Region.**

An inverse correlation was observed between the incidence of Grade 3 radiation-induced dermatitis and the concentration of CeO_2 nanoparticles given. Animals exposed to radiation combined with either 15 nM or 15 µM concentration of CeO_2 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. 7:1223-1231.*

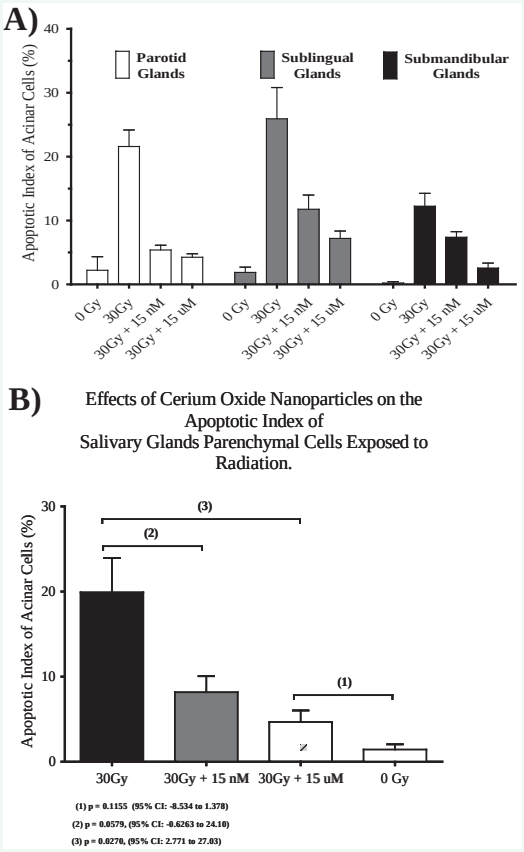

Figure 11 **Effects of Cerium Oxide Nanoparticles on the Apoptotic Index of Salivary Glands Parenchymal Cells After Radiation to the Head and Neck Region.**

(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 CeO_2 nanoparticles (5.32% and 4.25%, respectively). Non-radiated 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 CeO_2 nanoparticles resulted in a reduction in the magnitude of elevation to 11.8% and 7.2%, respectively after radiation. Non-radiated submandibular glands had a baseline apoptotic index of 0.2%. While radiation increased the index to 12.2%, by pre-treating with CeO_2 (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 CeO_2 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. 7:1223-1231.*

CeO_2 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 [40]. This study is the first to show that CeO_2 nanoparticles confer radioprotection on colon intestinal cells by exerting free radical scavenger properties and SOD mimetic properties.

CeO_2 Nanoparticles Reduce ROS levels and Protect Normal Human Colon Cells From Radiation-Induced Cell Death *in vitro*: In order to investigate the effects of CeO_2 nanoparticles on ROS production, normal human colon cells (CRL 1541) were exposed to increasing concentrations of CeO_2 nanoparticles 24 hours prior to a single exposure of 20 Gy radiation. ROS production was measured using the Image-iT LIVE™ green ROS detection kit. Results show that when radiation was administered as single therapy, the qualitative production of ROS was significantly increased. However, when CeO_2 nanoparticles were administered 24 hours prior to radiation, the presence of CeO_2 nanoparticles significantly 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 CeO_2 nanoparticles (Figure 12A) [21].

In another set of experiments, normal human colon cells (CRL 1541) were exposed to increasing concentrations of CeO_2 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 CeO_2 nanoparticles were administered 24 hours prior to radiation, the CeO_2 nanoparticles significantly protected the cells from radiation-induced cell death (3% for 1 nM, 1% for 10 and 100 nM) (Figure 12B) [21].

CeO_2 Nanoparticles Induce SOD-2 Expression in Normal Human Colon Cells *in vitro*: The effect of CeO_2 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 CeO_2 nanoparticles and in a dose-dependent fashion, the band intensity of SOD-2 in 100 nM CeO_2 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 CeO_2 nanoparticles (Figure 13) suggesting that CeO_2 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 CeO_2 nanoparticles [21].

CeO_2 Nanoparticles Reduce Apoptotic Cell Death in Gastrointestinal Mice Cells *in vivo*: In an attempt to investigate the ability of CeO_2 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 CeO_2 nanoparticles in combination with radiation exhibited a significant decrease in apoptotic colon cryptic cells (as measured by TUNEL) and Caspase-3 expression as compared to the colonic crypt cells from radiated (no CeO_2) 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 CeO_2 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 CeO_2 nanoparticles as compared to control (normal) mice which could be explained by the fact that CeO_2 may reduce the normal intrinsic cell death pathway and/or normal metabolic ROS, as reviewed by Rzigalinksi [7].

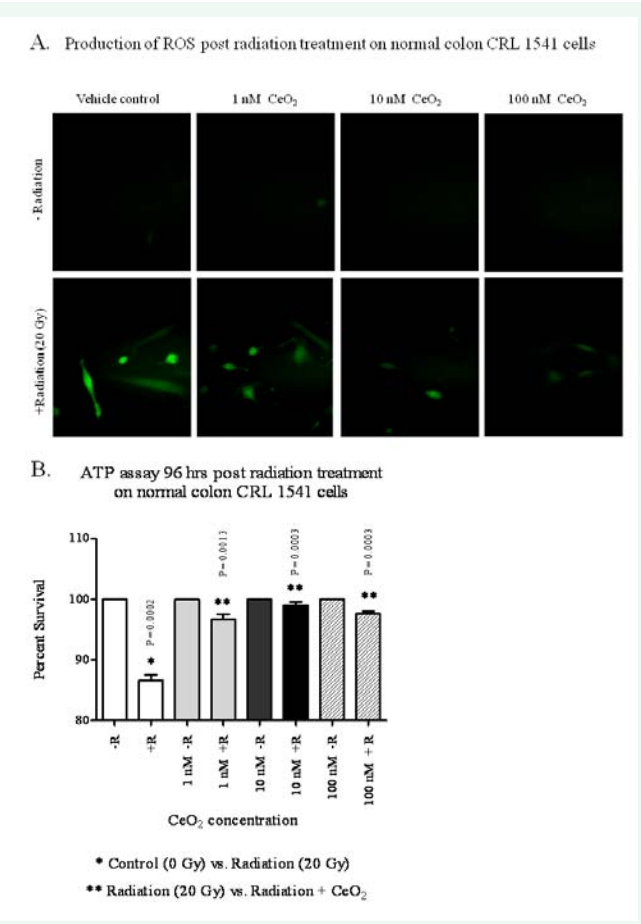

Figure 12 CeO_2 Nanoparticles Protect Normal Colon Cells Against Radiation-Induced Cell Damage.

CeO_2 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 CeO_2 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 CeO_2 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. 5:698-705.*

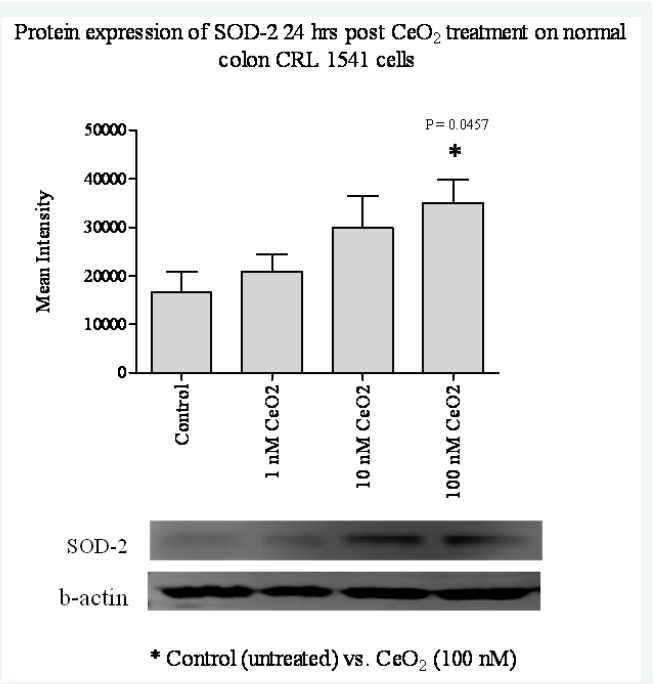

Figure 13 **CeO_2 nanoparticles induce protein SOD-2 expression.**

CeO_2 nanoparticles induce protein SOD-2 expression. The effect of CeO_2 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 CeO_2 nanoparticles. The protein band intensity of SOD-2 in cells incubated with 100 nM CeO_2 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. 5:698-705.*

To demonstrate the ability of the CeO_2 nanoparticles to induce the overexpression of SOD-2 colons from mice were sectioned 24 hours after a single injection of CeO_2 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 CeO_2 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 CeO_2 nanoparticles show an increase in SOD-2 expression [21].

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 quality of life 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 quality of life.

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 [41]. 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 [41]. 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 quality of life.

The above report lends a great deal of credence to the argument for the use of CeO_2 nanoparticles in a therapeutic setting as a free radical scavenger, especially in the context of therapeutic ionizing radiation. As mentioned above, CeO_2 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, mice administered with CeO_2 nanoparticles experience no serious

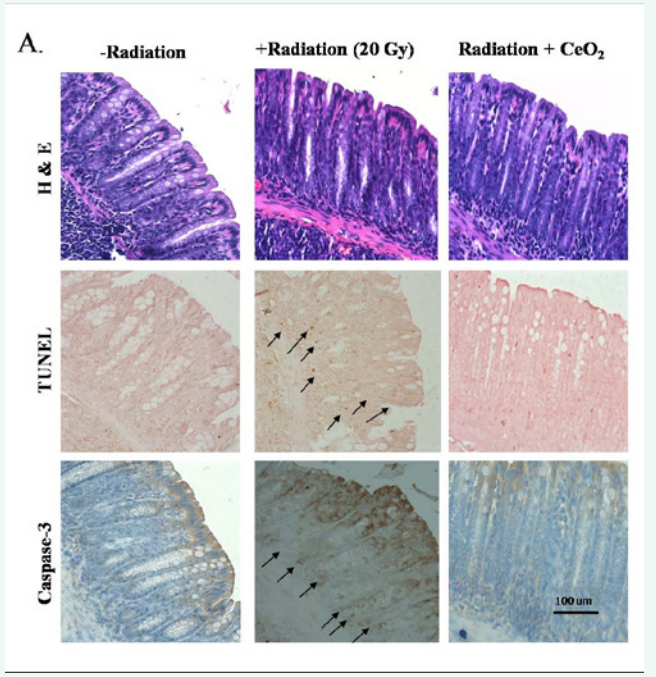

Figure 14 **CeO_2 Nanoparticles Protect Normal Human Colon Tissue from Radiation-Induced Cell Death.**

CeO_2 nanoparticles protect normal human colon tissue from radiation-induced cell death. Hematoxlin 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 CeO_2 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. 5:698-705.*

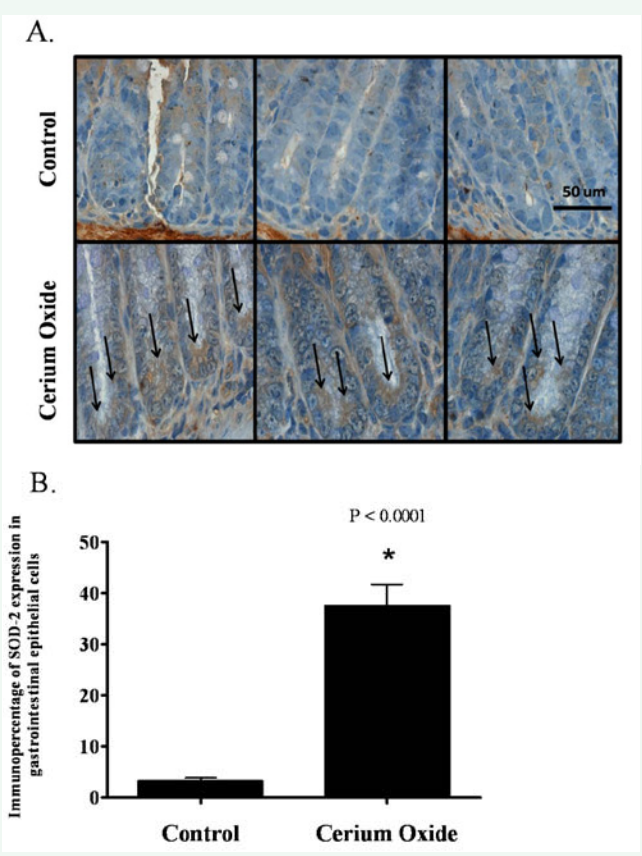

Figure 15 **CeO_2 Nanoparticles Induce SOD-2 Expression in Normal Colon.**

CeO_2 Nanoparticles induce SOD-2 expression in normal colon. **A**. Representative sections of SOD-2 expression (brown staining) in colonic crypts in mice treated with CeO_2 nanoparticles or in normal (control) mice. Colons were collected 24 hours post a single injection of CeO_2 nanoparticles. **B**. The immunopercentage of SOD-2 expression increased by 40% in mice treated with CeO_2 nanoparticles as compared to control mice. Each data point represents the mean +/- 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 5:698-705.*

side-effects, demonstrating the low toxicity of CeO_2 nanoparticles [20].

Elevated ROS levels have long been implicated in numerous diseases such as kidney fibrosis [42], chronic inflammation and organ dysfunction, especially when induced by ionizing radiation [43]. It is now widely accepted that ROS can interfere in intracellular processes which cause the above mentioned injuries. Thus, the therapeutic value of CeO_2 nanoparticles may be due to their free radical scavenging properties. Furthermore, CeO_2 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 Ce^{3+}/Ce^{4+}[7]. The *in vivo* experiments also reinforce the conclusion that CeO_2 nanoparticles confer significant protection from ionizing radiation as evidenced by TUNEL and Caspase-3 stains, indicators of cell apoptosis [44].

In the end, while CeO_2 nanoparticles may affect intracellular oxidative pathways, we show clearly that they 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 CeO_2 nanoparticles are at the forefront of the effort to utilize emerging nanotechnology to improve quality of life and healthcare, and that they hold great potential for future clinical trials.

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