Vacancy Engineered Ceria Nanostructures for Protection from Radiation-induced Cellular Damage.

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ABSTRACT

The ability of engineered cerium oxide nanoparticles to confer radioprotection was examined. Human normal and tumor cells were treated with nanoceria, irradiated and cell survival was measured. Treatment of normal cells conferred almost 99% protection from radiation-induced cell death while the same concentration showed almost no protection of tumor cells. For the first time, nanoceria is shown to confer radioprotection to a normal human breast line but not to a human breast tumor line, MCF-7.

Radiation therapy is one of the most widely utilized procedures for the treatment of cancer. While it is quite efficient at reducing and eliminating cancer cells, the normal cells in close proximity to the treatment site are inevitably exposed to the harmful radiation. During the process, free radicals are formed through ionizing reactions that are then capable of destroying normal tissues. These newly formed free radicals react with both DNA and RNA, resulting in molecular alterations. When cells are exposed to radiation, the level of protective molecules released, such as superoxide dismutase (SOD), glutathione, and metallothionine increases and DNA repair mechanisms are intensified[1]. While the protective and repair mechanisms for cells are efficient, they are not capable of blocking or rectifying all of the damage. Those symptoms typically associated with radiation therapy (nausea, vomiting, fatigue, hair loss, etc.) result from the death of these normal tissues.

Ways in which normal cells can be protected from radiation while targeting the tumor cells has been an important area of focus since the 1950's. In an effort to combat these harmful effects of radiation therapy, various free-radical scavengers have been tested for their ability to protect normal cells. The most effective free-radical scavenger to date is 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[2, 3]. Although Amifostin is the only clinically relevant of these compounds, it suffers from a very short half-life in serum.

Cerium oxide is a rare earth oxide material from the lanthanide series of the periodic table. It is used in various applications, electrolytes for solid oxide fuel cells (SOFC)[4], ultraviolet absorbents[5], oxygen sensors[6, 7] and automotive catalytic converters[8]. Nanocrystalline cerium oxide (nanoceria) possesses some unique properties; blue shift in ultra violet absorption spectrum[5], shifting and broadening in Raman allowed modes[9] and lattice expansion[5, 10]. These unique properties of nanoceria are proven to be beneficial in the present applications and open avenues for plethora of newer applications.

We have observed that cerium oxide nanoparticles increase neuronal lifespan in culture[11]. Its micron counterpart does not have any effect on the cell survival. The biological activity of the cerium oxide nanoparticles was assessed in an organotypic tissue culture model of rat cells and it was observed that cerium oxide nanoparticles prolong brain cell longevity in culture, by 2-3 fold or more. Further, cerium oxide nanoparticles reduced hydrogen peroxide (H_2O_2) and UV light– induced cell injury by over 60%. We hypothesize that the unique structure of cerium oxide nanoparticles, with respect to valence and oxygen defects, promotes cell longevity and decreases toxic insults by virtue of its antioxidant properties.

Because of the potential of free radical scavenging compounds to act as radioprotectants, we wanted to see if cerium oxide nanoparticles could confer radio-resistance to normal cells during ionizing radiation treatment.

Ceria nanoparticles prepared by the microemulsion process results in ultra fine, non-agglomerated particles in the range of 2-5nm[12]. The HRTEM image **(inset Figure 1)** indicates the formation of uniformly distributed, non-agglomerated nanoparticles of cerium oxide in the range of 3-5nm. The broad peaks in the XRD spectrum in **Figure 1** confirms the nanocrystallinity and the peaks can be indexed to the cubic phase of ceria with fluorite structure as found in the literature[13].

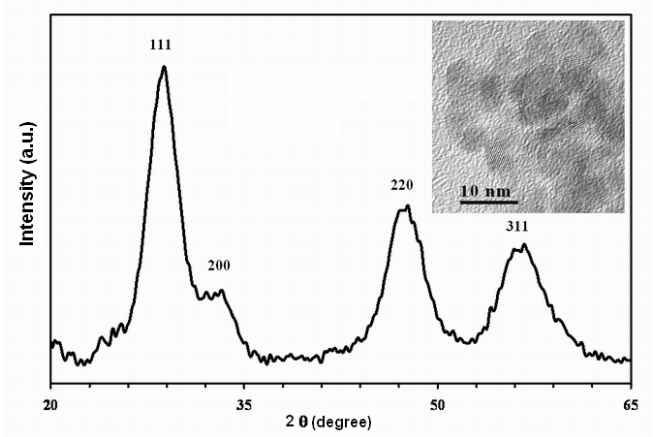

Figure 1. X-ray diffraction spectrum of the synthesized cerium oxide nanoparticles. (Inset) HRTEM image of the ceria nanoparticles.

The XPS spectrum **(Figure 2)** shows the presence of a mixed valence state (Ce^{3+} and Ce^{4+}) for the synthesized cerium oxide nanoparticles. The Ce^{3+} ions are introduced in nanoceria crystal lattice due to oxygen vacancies created by surface chemical reactions[14]. The concentration of Ce^{3+} ions in the cerium oxide nanoparticles varies with its particle size and its concentration is as high as 44% in the nanoparticles used in the present study[15].

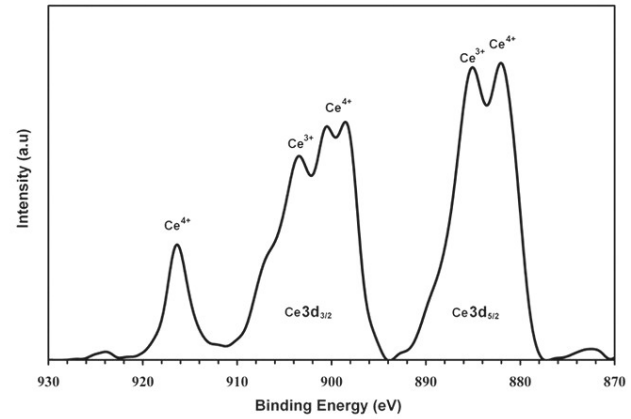

Figure 2. XPS spectrum of the synthesized cerium oxide nanoparticles showing presence of mixed valence (Ce^{3+} and Ce^{4+}) state.

MCF-7, breast carcinoma cell line was plated in 96 well plates. Plates were irradiated at the dose of 0 to 10 Gy, returned to a 37ºC incubator and viability measured at 24 and 48 hours by MTT assay. It was determined for MCF-7, human breast tumor cells, that 10 Gy irradiation and assay for viability at 48 hours reflected a greater than LD_{50} dose of ionizing radiation (**Figure 3**).

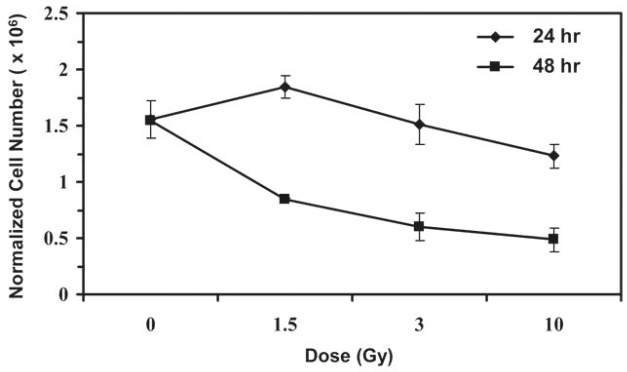

Figure 3. Dose response for ionizing radiation and effect on cell viability.

CRL-8798, an immortalized normal breast epithelial cell line, and MCF-7, breast carcinoma cell line were plated at 5,000 and 25,000 cells per well in a 96 well plate, respectively. Sterile filtered cerium oxide nanoparticles were added to each well at concentrations from 0 to 5 μM and viability was measured by MTT assay at 24 hours. Cerium oxide nanoparticles showed no significant toxic effect on CRL8798 and only a slight effect on MCF-7 cells at concentrations greater than 50 nM (**Figure 4**).

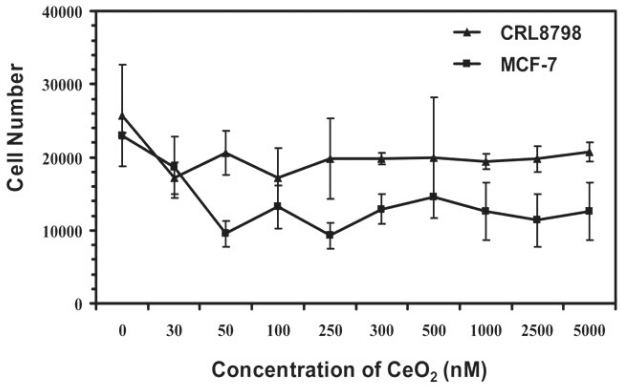

Figure 4. Cytotoxicity of cerium oxide nanoparticles on CRL8798 and MCF-7 cells in culture.

When cell lines were plated as in the above experiment and plates were irradiated with a dose of 10 Gy, both CRL8798 and MCF-7 were killed at level of 40-50%. When pretreated with 10 nM cerium oxide nanoparticles 24 hours prior to irradiation, CRL8798 cells were protected almost 100% whereas in MCF-7 cells, nanoceria showed no statistically significant protection from radiation-induced cell death (**Figure 5**).

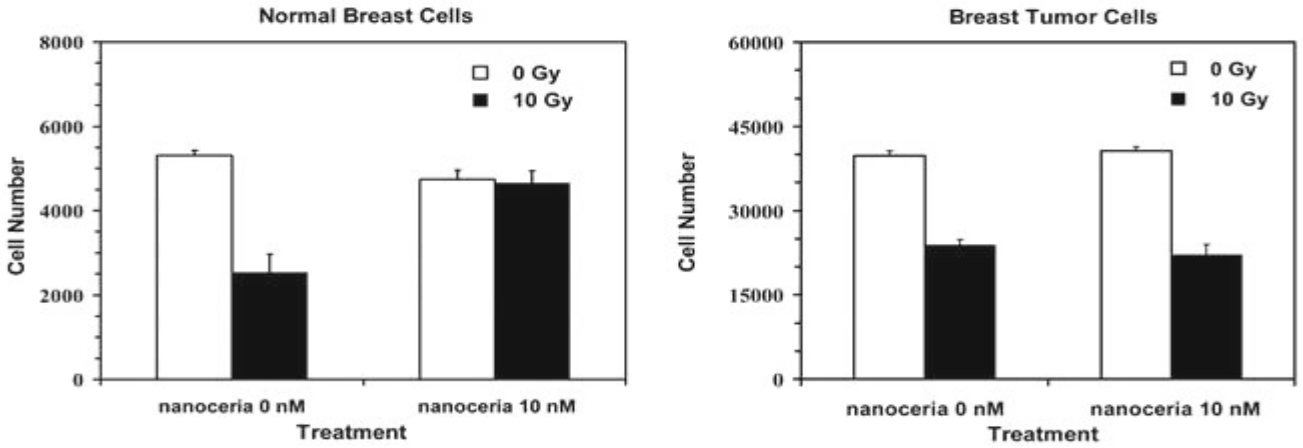

Figure 5. Cell viability of both CRL8798 and MCF-7 cells following 10 Gy irradiation either with or without 10 nM cerium oxide nanoparticles.

To look at the protective effect of cerium oxide nanoparticles on radiation-induced apoptotic cell death, cells were grown on chamber slides and pretreated with 10 nM nanoceria for 24 hours. Slides were irradiated with 10 Gy and incubated at 37ºC for 48 hours. Cell death, for adherent and non-adherent cells, was measured by TUNEL staining for both CRL8798 and MCF-7 cells. As seen with MTT cell viability assays, both CRL8798 and MCF-7 can be induced to undergo apoptosis following radiation exposure. When pretreated for 24 hours with 10 nM nanoceria, CRL8798 cells showed almost 100% protection from radiation-induced apoptosis whereas MCF-7 cells showed no statistically significant protection by nanoceria (**Figure 6, 7**).

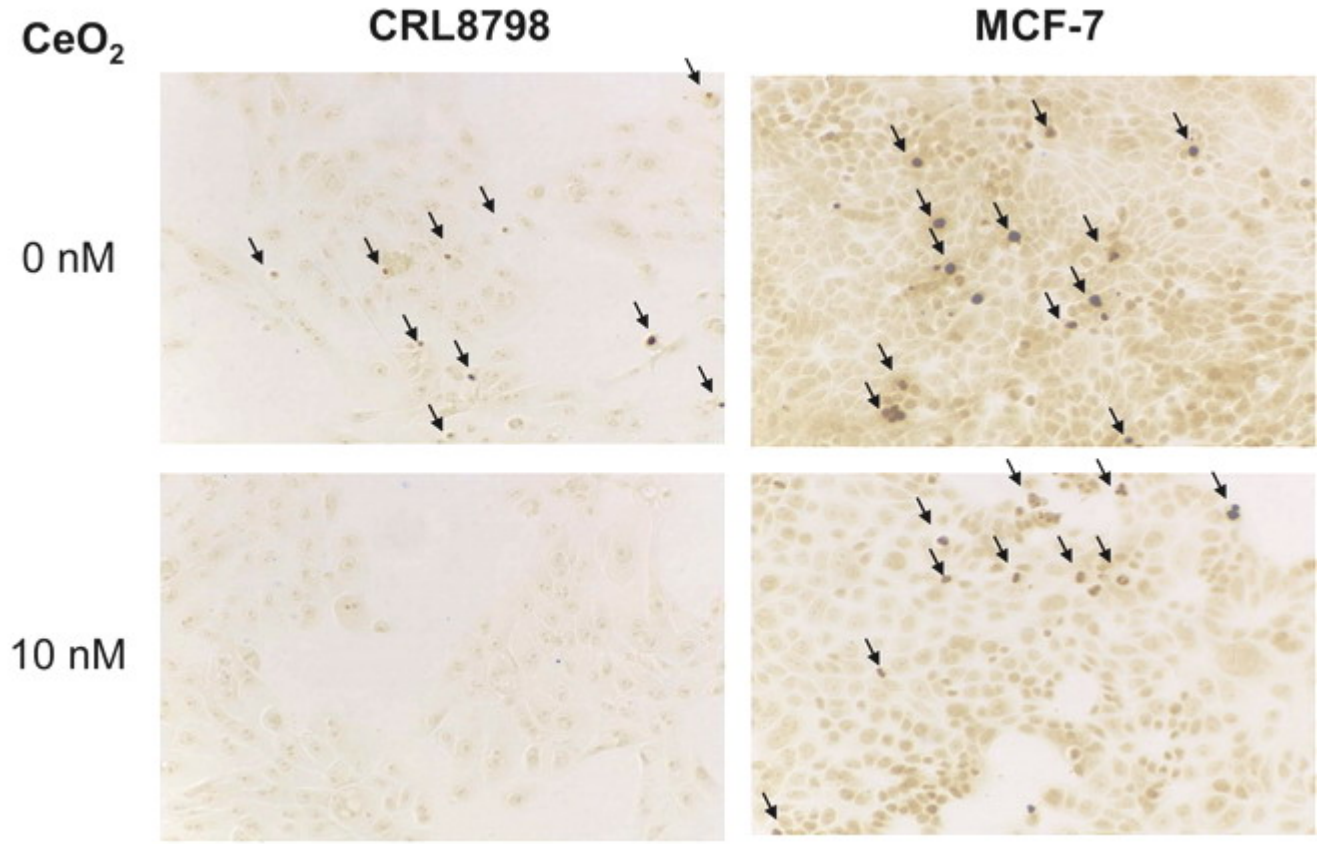

Figure 6. TUNEL staining of breast cells at 48 hours following 10 Gy irradiation and protection by cerium oxide nanoparticles. Arrows denote TUNEL positive apoptotic nuclei.

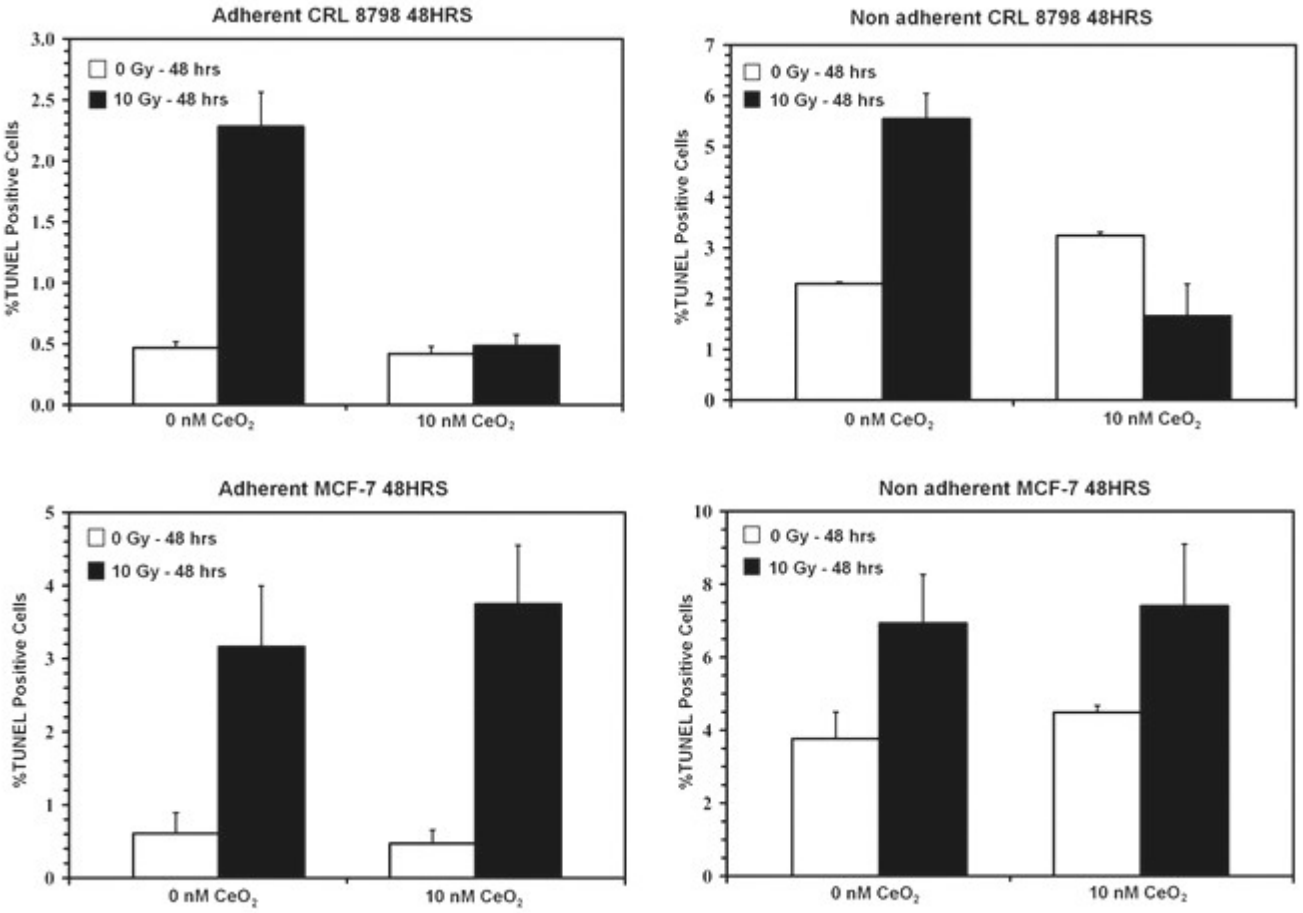

Figure 7. Protective effect of 10 nM cerium oxide nanoparticles on CRL8798 cells from radiation-induced apoptosis versus MCF-7 cells in culture. TUNEL staining was done for both adherent and non-adherent cells.

Effective long-lived radioprotectants with clinical applications are not well represented in most formularies. Amifostine is the only clinically relevant radioprotectant and it was synthesized in the 1950's at the Walter Reed Army Research Facility. Nanoceria shows very promising characteristics that may allow it to be a useful radioprotectant. Our studies have demonstrated a differential efficacy in normal versus tumor cells in culture. This could be due to several reasons such as differential uptake, differential intercellular activity or differences in chromatin structure or free-radical targets. Unpublished data in our lab has demonstrated that the nanoceria is taken up at the same rate by both normal and tumor cells. Inter- and extracellular pH differences are present in tumors versus normal tissue due to metabolic activity of the tumor cells[3]. These effects might have effect on the free-radical scavenging activity of the cerium oxide nanoparticles. Tumor cells tend to have more relaxed chromatin structure that can expose more bases as targets for free-radical attack[3]. A similar concentration of scavenger protecting fewer sites in normal cells versus the more relaxed chromatin and greater number of targets of tumor cells could help account for this observed difference.

We propose that nanoceria acts as an antioxidant due to the presence of the mixed valence states of Ce^{3+} and Ce^{4+} on the surface, (induced by the oxygen vacancies). By changing its oxidation state from Ce^{3+} to Ce^{4+} ceria nanoparticles scavenge the free radicals generated by irradiation. Another complex set of surface chemical reactions may be involved in renewing the oxidation state from Ce^{4+} to Ce^{3+}. We believe that perhaps there is an auto-regenerative reaction cycle ($Ce^{3+} \rightarrow Ce^{4+} \rightarrow Ce^{3+}$) continuing on the surface of ceria nanoparticles and this may be the mechanism by which it provides the material with an unprecedented antioxidant activity. The auto-regenerative anti-oxidant property of these nanoparticles appears to be a key component of its radioprotective action.

Cerium oxide nanoparticles have very low or no toxicity based on our cell culture data as well as the available literature[16, 17]. Furthermore, cerium oxide nanoparticles are long-lived and can confer their beneficial effect for extended periods of time without re-dosing. Shortcomings of amifostin are: very short half-live of less than 10 minutes in serum, toxicity at higher doses and toxicity based on route of administration[18].

Taken together, these data suggest that cerium oxide nanoparticles could have a role as an effective radioprotectant for normal tissues as well as show a differential protection in normal cells as compared to tumor cells. Further studies will determine the mechanism of this differential effect as well as determine the efficacy in animal models of ionizing radiation, both for general radioprotection as well as for radiation oncology applications.

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Supporting Information Available. Materials and Methods.

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Cell Lines and Reagents. CRL-8798, an immortalized normal breast epithelial cell line, and MCF-7, breast carcinoma cell line, were obtained from American Type Culture Collection (Manassas, VA). Cell lines were maintained in a growth medium comprised of the following constituents: minimum essential medium (Eagle) with 2mM l-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% from Invitrogen Corporation (Carlsbad, CA). The cells were incubated at 37°C, and 5% CO_2.

Cerium Oxide Nanoparticle Synthesis and Characterization. Cerium oxide nanoparticles were prepared by the microemulsion method. The nano-sized micelles in the microemulsion system act as nano-reactors for the nanoparticle formation. The microemulsion system consisted of the surfactant, sodium bis(2-ethylhexyl) sulphosuccinate (AOT), toluene and water. All the chemicals were purchased from Aldrich Chemicals Company, Inc. Details of the synthesis are published elsewhere[1, 2]. The particles obtained in toluene were precipitated and washed with acetone and water for several times to remove the surfactant completely from the particles. The particles thus obtained were then re-dispersed in water. The solution was sterile filtered through a 0.2 μm syringe filter for use in radioprotection experiments.

The particle morphology was studied using high resolution transmission electron microscopy (HRTEM), x-ray diffraction (XRD) was used for crystal structure studies and the surface chemistry of the ceria nanoparticles was studied using x-ray photoelectron spectroscopy (XPS). The Ceria nanoparticles were deposited on the carbon coated copper grid for HRTEM analysis by the dip coating method. The HRTEM images of the particles were obtained with a Philips (Tecnai Series) transmission electron microscope operating at 300 keV. The crystal structure information of the synthesized nanoceria was taken using XRD (Rigaku model) with Cu $K_{\alpha 1}$ radiation. The data was recorded at scan rate of 0.25 degree/min. The XPS data was obtained using a 5400 PHI ESCA (XPS) spectrometer. The base pressure during XPS analysis was 10^{-9} Torr and Mg-K_{α} X-ray radiation (1253.6eV) at a power of 200 watts was used. The binding energy of the Au ($4f_{7/2}$) at 84.0±0.1 eV was used to calibrate the binding energy scale of the spectrometer. Any charging shift produced in the spectrum by the sample was carefully removed by taking C (1s) position (284.6 eV) as a reference line as shown by Barr and Seal[3]. XPS spectra smoothening and baseline subtraction was carried out using PeakFit (Version 4) software.

Radiation Treatment. Cell lines were plated at 5,000 to 1.3 x 10^6cells per well in 96 or 6 well plates. Sterile filtered Cerium Oxide nanoparticles were added to each well at 10 or 100 nM, 24 hours prior to irradiation. Plates were irradiated at the dose of 0 to 10 Gy with a Varian 600C Single Beam Linear Accelerator (Palo Alto, CA). Plates were returned to an incubator and assayed for cell survival at 24 and 48 hours after irradiation.

MTT Assay. Cell viability was assayed by MTT assay as described in the manufacturer's protocol. Briefly, a 20 μl of a 5mg/ml solution of MTT (Promega Corporation, Madison, WI) was added to each well of a 96-well plate, 2 hours before the end of the incubation. The plates were incubated in a CO_2 incubator at 37ºC for 2 hours. The supernatant was aspirated and the cells dissolved in 200 μl of DMSO by pipetting up and down. Plates were further incubated at room temperature for 2 hours. The plates were transferred to a plate reader and absorbance measured at 550nm.

TUNEL Assay. Cells were grown on chamber slides (Fisher Scientific, Hampton, NH) and pretreated with 10 nM nanoceria for 24 hours. Slides were irradiated with 10 Gy and incubated at 37ºC for 24 or 48 hours. TUNEL staining was carried out as per the manufacturer's protocol (Chemicon International, Temacula, CA). Briefly, both slide adherent and suspended cells were collected and assayed for TUNEL staining by first fixing in 1% paraformaldehyde. Endogenous peroxidase was quenched with 3% H2O2 for 5 minutes followed by washing in dH2O for 5 minutes. Slides were incubated with terminal deoxynuclotidyl transferase with digoxigenin-linked dUTP for 1 hour at 37ºC. The reaction is stopped, slides washed and then incubated with anti-digoxigenin antibody conjugated with horseradish peroxidase for 30 minutes at room temperature in a humidified chamber. Slides were washed, incubated with the peroxidase substrate for 3-6 minutes, washed, coverslips mounted and viewed under a light microscope.

Statistical Analysis. All experiments were completed in triplicate and results expressed as Mean +/- SD. Differences in radiosensitivity of cell lines and radioprotection of compounds was determined by a paired Student's T-test and a p-value equal to or less than 0.05 was considered statistically significant.

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