Use Of Cerium Oxide Nanoparticles For Protection Against Radiation-induced Cell Death

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USE OF CERIUM OXIDE NANOPARTICLES FOR PROTECTION AGAINST RADIATION-INDUCED CELL DEATH

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ABSTRACT

The ability of engineered cerium oxide nanoparticles to confer radioprotection was examined. Rat astrocytes were treated with cerium oxide nanoparticles to a final concentration of 10 nanomolar, irradiated with a single 10 Gy dose of ionizing radiation and cell death was evaluated by propidium iodine uptake at 24 and 48 hours after radiation insult. Treatment of rat astrocytes with nanoceria resulted in an approximate 3-fold decrease in radiation induced death. These results suggest that the nanoceria are conferring protection from radiation induced cell death. Further experiments with human cells were conducted. Human normal and tumor cells (MCF-7 and CRL8798) were treated with the same dosage of cerium oxide nanoparticles, irradiated and evaluated for cell survival. Treatment of normal cells (MCF-7) conferred nearly 99% protection from radiation-induced cell death while the same concentration of nanoceria showed almost no protection in tumor cells (CRL8798). TUNEL analysis results of similarly treated cells demonstrated that nanoceria reduced radiation-induced cell death by 3-fold in normal breast cells but not in MCF-7 tumor cell lines when cultured under the same conditions. We concluded that cerium oxide nanoparticles confer radioprotection in a normal human breast line (CRL 8798) but not in a human breast tumor line (MCF-7). It is hoped that the outcome of this study will guide future endeavors toward a better elucidation of the molecular pathways involved in the protection of cells with nanoceria against radiation-induced cell death, as well as the minimization of the bystander effect in radiation therapy.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ACRONYMS/ABBREVIATIONS

SOFC	Solid Oxide Fuel Cells
SOD	Superoxide Dismutase
RNA	Ribonucleic Acid
HRTEM	High Resolution Transmission electron microscopy
XRD	X ray diffraction
UV	Ultra violet
AOT	Sodium bis (2-ethylhexyl) sulphosuccinate
MV	Mega Volts
Gy	Grey
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
ATCC	American Tissue Culture Collection
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
DAB	Diaminobenzidine
HRP	Horseradish peroxidase
ICP/MS	Induced coupled plasma/mass spectroscopy
BCA	Bicinchonic Acid
PPM	Parts per million
DNA	Deoxyribonucleic acid

INTRODUCTION

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. The actual damage to DNA may take several forms. There may be change or loss of a base, rupture of hydrogen bonds between DNA strands, dimerization, cross link formation, single strand breaks and/or double strand breaks. On the chromosomal level, post-radiation abnormalities are usually due to chromosome breaks and error in rejoining post-replication chromosomes. These events may lead to improper segregation on chromosomes during mitosis causing radiation induced mitotic death (mitotic catastrophe). 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 (the bystander effects), 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.

Rzigalinski et al. 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%. They 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-protection in normal cells during ionizing radiation treatment.

Rationale and Objectives

Data supporting the antioxidant properties of cerium oxide nanoparticles is mounting. Many studies suggest that nanoceria act as free radical scavengers and may render protection against chemical, biological and radiological insults that promote the production of free radicals. Skepticism regarding the properties of nanoceria to scavenge free radicals in a biological system is a result of a limited research done on this area. Therefore, consistent studies that address the free radical scavenging properties of nanoceria in a biological system are urgently needed. In an attempt to investigate the properties of nanoceria in scavenging free radicals in response to radiation, the objectives of this study were as follows: 1) to observe the radioprotective effects, if any, of nanoceria in normal rat astrocytes 2) to determine if the radioprotective effects observed in normal rat astrocytes could be mimicked in human cell lines 3) to determine if nanoceria exhibits similar radioprotection against ionizing radiation in human normal and tumor breast cell lines 4) to evaluate any nanoceria uptake in normal versus tumor breast cell types in order to determine if there is any differential uptake.

It is hoped that the outcome of this study will guide future endeavors toward a better elucidation of the molecular pathways involved in the protection of nanoceria against radiation-induced cell death, as well as the minimization of the bystander effect in radiation therapy.

MATERIALS AND METHODS

Rat astrocytes experiments

Materials

Pregnant Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). Dulbecco's modified essential medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), Fetal bovine serum, Penicillin-Streptomycin, L-Glutamine, horse serum, 0.25% Gibco trypsin and Fatty acid free albumin were obtained from Invitrogen (Carlsbad, CA, U.S.A.). L-glutamate acid, , D-Glucose, and Propidium iodide (Pr I) were purchased from Sigma (St. Louis, MO, U.S.A.). Hard Plates were from Flexcell International (Hillsborough, NC, U.S.A.). The Orbital Shaker was obtained from Bellco Glass (Vineland, NJ, U.S.A.). Microplate reader and Auto strip washer were purchased from Bio-Tek Instruments (Winooski, VT, U.S.A.).

Cell Culture Astrocyte and Mixed Cell Dissection

Mixed cultures of neuronal plus glial cells were initially prepared form 1–2-day-old neonatal Sprague-Dawley rat pups. Pups were sacrificed by decapitation and the neocortices were rapidly dissected in a sterile hood. First, the brains were removed and placed in a Petri dish containing sterile dissecting saline (137 mM NaCl, 5.3 mM KCl, 0.17 mM $Na_2HPO_4.7H_2O$, 0.22

mM KH_2PO_4, 10 mM HEPES, 33 mM Glucose). Cerebral hemispheres, olfactory bulbs, hippocampal formations, basal ganglia and meninges were removed. The cleaned cortices were removed and placed in a second Petri dish containing fresh dissecting saline. Care was taken to avoid contamination of cortices by the olfactory bulb or lower brain stem.

Cell Preparation

The isolated cortices were moved to a 60 mm dish with 5 mL dissecting saline containing trypsin (0.25% in Hanks Gibco, fully sterilized) for 10 min. Cortical tissue was gently diced into small pieces, and transferred to a tube containing 5 mL astrocyte feed medium, containing Dulbecco's modified essential medium (DMEM), 10% fetal bovine serum (FBS), Penicillin-Streptomycin (prepared with 5,000 U/mL penicillin G sodium with 5,000 ug/mL streptomycin sulfate in 0.85% saline), and 2 mM L-glutamine. The tissue was homogenized and the suspension was centrifuged for 5 min at 2,000 x g to collect tissue and cells. The supernatant was removed and 5 mL of fresh culture medium was added for a second wash. After washing, the supernatant was removed and 5 mL of culture medium was added and tissue fragments were homogenized. The tissue suspension was further dissociated by triturating with a series of flame – narrowed glass pipettes. The suspension was then filtered with a 70-µm nylon cell strainer to remove undissociated tissue. The filtrate, containing the suspended cells, was diluted with astrocytes media. Cell counts were performed on a 10 µL sample using a hemocytometer, and the cell suspension was adjusted to contain 8.0×10^6 cells/mL. These flasks then contained mixed organotypic brain cell cultures. All cell cultures were maintained in a 5% CO_2 incubator at 37°C.

For the flask cultures, 15 mL of medium was removed from each 150-cm2 flasks and same amount of growth medium, astrocyte feed medium (DMEM, Penicillin-Streptomycin, L-glutamine and 10% FBS), was added. Media was changed every 2 to 3 days after preparation.

Preparation of astrocytes cultures

To prepare pure enriched astrocyte cultures, the flasks containing mixed organotypic cultures are treated with trypsin (0.25% trypsin and 0.02% EDTA in saline), which detaches the cells from the bottom of the flasks. In brain cell cultures, only the astrocytes and microglia survive the trypsinization process. Since microglia have already been removed from these cultures, preparation of pure astrocyte cultures is possible.

After 14 days cultivation, flasks from which microglia have been removed were used to subculture pure astrocytes. The existing culture medium in flask was removed and flasks were washed once with 12 mL plain DMEM (no calf serum, glutamine, pen/strep). 12ml fresh plain DMEM was added and then flasks were incubated for 5 minutes. After incubation, for each 150 cm^2 flask, 10 mL 0.25% trypsin in EDTA was added. Flasks were kept lying flat in the incubator at 37° C, for 2 minutes. After 2 minutes, the cells had not yet detached, but were loosened from the flask. The trypsin solution was aspirated by vacuum. Then 12 mL DMEM without calf serum was added and flasks were incubated at 37° C for 10 minutes, sufficient time to lift the cells from the flask bottom. Cells were detached from the flask bottom after 10 minutes incubation. The flasks were sharply whacked on the bottom to help remove cells. The medium, containing the astrocytes, was decanted into a sterile 50 mL conical tube. Then the flasks were rinsed with 12 mL DMEM and the wash was added to the 50 mL conical tubes (two flasks/tube). The cell

suspension was centrifuged at 1500 x g for 7 minutes to pellet cells. The supernatant was removed and 5mL of culture medium was added to resuspend and break up cell pellets by gently pipetting up and down 10-20 times using a 5 or 10 mL pipette. Next, all astrocyte suspensions were combined into one 50 mL tube. Cell count was performed by a hemocytometer using a dilution of 0.9 mL media to 0.1 mL cell suspension. The cell suspension was adjusted to contain $1.5x\ 10^{6}$ cells/mL. $1.5x10^{6}$ cells were seeded into each well of a collagen coated plate. Cell cultures were maintained in a 5% CO2 incubator at 37°C. Cells were confluent and ready for experiments after 14 days cultivation.

Propidium Iodide (Pr I) Uptake

Injury was assessed by measuring propidium iodide (Pr I) uptake. Propidium iodide (Pr I) is used as a detection method for live/dead cells. The dye is fluorescent orange with a molecular weight of 792. Due to its size, it is normally excluded from cells with intact plasma membranes. But when cells begin to die, their plasma membrane compromised and becomes leaky. This allows Propidium iodide (Pr I) to enter the cells, where it intercalates with DNA in the nucleus, staining the nucleus a fluorescent red. Therefore, dead or dying cells can be detected and enumerated by Pr I staining. The culture media from the wells of the tissue culture plates used for experiments were decanted and replaced with 1% FAF-DPBS (fatty acid free albumin-Dulbecco's phosphate buffered saline). Then 10 uL propidium iodide (1%) was added to each well. Cells were returned to the incubator for 15 min after addition of propidium iodide (Pr I). In order to focus using the fluorescent microscope with water immersion lens, there needs to be a

fluid level of 2.5 mL in each tissue culture well. So, after the 15 minutes incubation, 1.5 mL of the 1% FAF-DPBS was added to adjust the well volume to appropriate levels. Fluorescent stained nuclei were counted using a Zeiss epifluorescence microscope. Pr I-stained cells were quantified using an imaging program (Felix 32 software). Ten fields (there is a 100 square grid in the fluorescent microscope lens) were randomly counted within the grid. The count in 10 fields was averaged to determine the average Pr I uptake per field for that well.

Human cells experiments

Materials

Cancer cell lines HTB-22 (MCF-7) and CRL-8798 were obtained from ATCC cell line collections (Manassas, VA, USA). Dulbecco's modified essential medium (DMEM), Minimum enriched medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), Fetal bovine serum, Sodium Pyruvate 100 mM, Sodium Bicarbonate Solution 7.5% (w/v), MEM Non-essential Amino acids, L-Glutamine, horse serum, 0.25% Gibco trypsin were obtained from Invitrogen (Carlsbad, CA, U.S.A.). MEGM Mammary Epithelium Growth Medium complete with 10 ng/mL hEGF, 5 ug/mL Insulin, 0.5 ug/mL Hydrocortisone, Gentamicine and Amphotericin-B was obtained from Clonetics Cambrex (Walkersville, MD, USA). Insulin Cat. No. I-0516, cholera toxin A subunit Cat. No. C-8180, holo-Transferrin Cat No. T1283, Pluronic F-68 Solution (10%) Cat No. P5556, HEPES Cat. No. H-3375 and Propidium iodide (Pr I) were

purchased from Sigma (St. Louis, MO, U.S.A.). Reagent alcohol (A962-4), Sodium Hydroxide 10 N solution, 30% w/w (SS255-1) and paraformaldehyde (UN2213) were obtained from Fisher chemicals (Fairlawn, NJ, USA). Multiwell Primaria 6 well plates were from Becton Dickinson Labware (Franklin Lakes, NJ, U.S.A.). T-75 cell culture flask and Costar 3595 96 well plates were obtained from Corning Incorporated (Corning, NY, U.S.A.). MTT Cell Proliferation Assay was obtained from ATCC (Manassas, VA, USA). Microplate reader and Auto strip washer were purchased from Bio-Tek Instruments (Winooski,VT, U.S.A.). Ionizing radiation was induced using a single energy linear accelerator Varian 600 C at the MD Anderson Cancer Center in Orlando Regional Healthcare (Orlando, Fla, USA)

Cell culture

CRL 8798 were routinely maintained in a monolayer culture in vitro at 37 degrees Celsius in 5% CO2 in MEGM (Mammary Epithelial Growth Medium, serum free) Clonetics supplemented with 0.005 mg/mL transferring (Sigma) and 1 ng/mL cholera toxin (sigma). HTB-22 (MCF-7) were routinely maintain in a monolayer culture in vitro at 37 degrees Celsius in 5% CO2 in minimum growth medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1mM sodium pyruvate and supplemented with 0.01 mg/mL bovine insulin and 10% fetal bovine serum. All cell lines were allowed to reach 80-90 % confluence in 75 cm2 flask at 37 degrees Celsius and 5% CO2 and harvested 24 hours later, discarding the dead before plating the 96 well and 6 well plates for the experiments.

Cell preparations

To prepare the 6 well and the 96 well plates cultures, the flasks containing cancer cell lines are treated with trypsin (0.25% trypsin and 0.02% EDTA in saline), which detaches the cells from the bottom of the flasks. The existing culture medium in flask was removed by aspiration. 5 mL of trypsin-EDTA is added to each flask and incubated at 37 degrees Celsius until cells are dispersed. The cell suspension containing trypsin is transferred to a 15 mL conical tube and 5 mL of media is added to neutralize the trypsin. The cell suspension was centrifuged at 1000 x g for 1 minute to pellet cells. The supernatant was removed and 5 mL of culture medium was added to resuspend and break up cell pellets by gently pipetting up and down 10-20 times using a 5 mL pipette. Cell count was performed using a KOVA Glasstic Slide 10 by HYCOR (Garden Grove, CA, USA). The cell suspension was adjusted to contain 50,000 cells/100 uL. Five thousands cells were seeded into each well of the 96 well plate and 500,000 into each well of the 6 well plates. Cell cultures were maintained in a 5% CO2 incubator at 37°C.

Preparation of CeO2 working solution

Cerium oxide nanoparticle synthesis and characterization.

Cerium oxide nanoparticles were a gift from Dr. Seal laboratory at the AMPAC facility at the University of Central Florida, Orlando, FL. A working solution of 200 nM of cerium oxide was prepared in Ca++ and Mg++ free PBS from the stock solution (0.005 M) of cerium oxide (particle size 5-10 nm). The working solution was filtered sterilized through a 2 um filter and stored at 4°C.

Radiation experiment

Experiments involving ionizing radiation were conducted in 96 well plates and 6 well plates. A radiation dose of 10 Gy/4.5 mins (photons: 6 MV) was applied to each plate using a Varian 600C single energy linear accelerator. Following irradiation, all cultures were returned to the humidified incubator at 37°C and 5% CO2. Sham irradiated plates were used as non irradiated controls.

Trypan Blue exclusion determinations

Trypan Blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. Cells were grown in 6 well plates, irradiated and incubated for 24 and 48 hrs in the incubators. After incubation, cell were detached by trypsinization and placed in 0.5 mL of media (dilute cells in complete medium without serum to an approximate concentration of 1 x 10^5 to 2 x 10^5 cells per mL) in a screw cap test tube. Next, 0.1 ml of 0.4% Trypan Blue Stain was added and mixed thoroughly. The mix was allowed to stand for 5 min at 15 to 30°C (room temperature). After five minutes incubation at room temperature the cells were counted in the hemocytometer. Using a microscope, cells were observed and non-viable and viable cells were counted. Cell viability was determined by the following formula: [(Total cells counted – stained cells) / Total cells counted] x 100. All results are expressed in percent control.

MTT cell proliferation assay

The cell proliferation assay was performed according to the protocol of the commercially available MTT cell proliferation assay kit (ATCC Cat. No. 30-1010K Manassas,VA,USA) with out any modifications. Briefly, approximately 5,000 cells were plated in a 96 well plate 24 hr before the experiment to permit the cells proper attachment. After irradiation the plates were returned immediately to the incubator. After incubation (24h, 48h) 10 uL of the MTT reagent was added to each well and the plates were returned to the incubator. After 4 hours incubation,

100 uL of detergent reagent was added to each well and the plate was incubated at room temp in the dark for 4 hrs. Absorbance at 562 nm was read using a BioTek ELx 808 Ultra Microplate reader (BIO-TEK instruments, Inc. Winnoski, VT, USA). Cell proliferation was determined using a standard curve. All results are expressed as percent control.

Dead End Colorimetric TUNEL System

The DeadEnd Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

Reagent preparation

4% Formaldehyde

A volume of 100 mL Phosphate Buffered Saline (PBS) was pour into the conical flask containing 4 grams of paraformaldehyde. The flask was covered with parafilm and transferred to

the fume hood. The flask was placed on top of the hotplate/stirrer inside the fume cupboard and set the heat control to 7 with moderate stirring. The solution was allowed to warm up till it turned from being cloudy to clear. When the paraformaldehyde was dissolved, the solution was let to stir and cool down. When cooled, the solution was transfer to a 4°C refrigerator.

Fixing Adherent cells on glass slides for microscopic analysis

Cells were grown on Lab-Tek Chamber Slides (50,000 cells per chamber) and let to reach 90% confluency before perform the experiment. A cerium oxide nanoparticles solution was added to reach a final concentration of 10 nanomolar, 24 hours before radiation. Following radiation, slides were incubated at 37°C, 5% CO2 for 24 and 48 hours. Then the supernatant was removed and suspended cells were removed and processed for TUNEL labeling according to protocol. The slides with attached cells were washed by dipping twice in a coplin jar containing PBS. A fresh jar of PBS was used for each wash. To fix the cells, the slides were immerse in a Coplin jar containing fresh 4% formaldehyde/PBS at 4°C for 25 min. Then the slides were washed three times with PBS and finally stored immerse in 70% ethanol at 4°C. The percentage of TUNEL positive cells from suspended and adherent cells was totaled.

rTdT Reaction mix

For each reaction, 98 uL of equilibration buffer was mixed with 1 uL of biotinylated nucleotide mix and 1 uL of rTdT enzyme in an eppendorf tube and kept on ice.

2X SSC

The 20X SSC solution was diluted 1:10 with deonized water and kept in a coupling jar at room temp.

0.3% Hydrogen Peroxide solution

To block endogenous peroxidases a 0.3 % hydrogen peroxidase was prepared by adding 150 uL of hydrogen peroxide to 50 mL of phosphate buffered saline and kept in a couplin jar at room temp.

Streptavidin HRP solution

The streptavidin HRP solution was diluted 1:500 by adding 2 uL of streptavidin HRP stock solution to 1000 uL of phosphate buffered saline in an eppendorf microcentrifuge tube and kept at room temp.

Diaminobenzidine solution (DAB)

The DAB components was prepared no more than 30 minutes prior to use and kept in the dark. Fifty microliters of the DAB substrate 20X buffer was added to 950 uL of deionized water. Then 50 uL of the DAB 20X chromogen and 50 uL of hydrogen peroxide 20X. One hundred microliters of the DAB solution was required per each slide.

Assay procedure

The TUNEL assay kit DEAD END Colorimetric TUNEL system (Cat No. G7130) was obtained from Promega. The assay was performed according to the manufacturer protocol. For apoptosis detection, cells were culture in 2 chamber Lab-Tek slides at a density of 50,000 cells until reaching 80-90% confluency. Cerium oxide nanoparticles were added to obtain a 10 nM concentration 24 hour prior to ionizing radiation exposure. After a 24 and 48 hour post-radiation incubation, cell were fixed by immersing slides in 4% paraformaldehyde solution in a coplin jar for 25 minutes at room temperature. Slides were washed by immersing in fresh PBS for 5 minutes at room temperature. Cells were permeabilized by immersing the slides in 0.2% Triton® X-100 solution in PBS for 5 minutes at room temperature. After permeabilization, slides were rinsed by immersing in fresh PBS for 5 minutes at room temperature two times. The excess liquid was removed by tapping the slides and were covered with 100 µL of equilibration buffer and equilibrated at room temperature for 5-10 minutes. After equilibration the slides were blotted

around the equilibrated areas with tissue paper and 100 µL of rTdT reaction mix was added to the sections on a slide. The sections in the slides were covered with plastic coverslips to ensure even distribution of the reagent and incubated at 37°C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur. After 60 minutes incubation, the plastic cover slides were removed and the reaction was terminated by immersing the slides in a 2X SSC solution in a coupling jar for 10 minutes at room temperature. After rinsing the slides, the slides were washed by immersing the slides in fresh PBS for 5 minutes at room temperature twice to remove unincorporated biotinylated nucleotides. The endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide in PBS for 3.5 minutes at room temperature. The slides were washed by immersing in PBS for 5 minutes twice at room temperature. After washing the slides, 100 µL of a diluted Streptavidin HRP solution (1:500) was added to each slide and incubate for 30 minutes at room temperature. After the incubation, the slides were washed by immersing in PBS for 5 minutes at room temperature. Then 100 µL of DAB solution was added to each slide and let to develop for approximately 10 mins or until there is a light brown background. After color development the slides were rinsed in deionized water several times. The slides were mounted in 100% glycerol and staining was observed under a light microscope. The percentage of TUNEL staining was deter mined by the following formula: stained cells (TUNEL positive) / total cell per field * 100.

Induced coupling plasma mass spectroscopy ICP/MS

The intracellular nanoceria concentration was determined by Induced coupled plasma mass spectroscopy (ICP/MS). The ICP/MS values were normalized according to the protein concentration obtain from the samples.

Sample preparation

In brief, the cells were plated in 6 wells plates as described before. One hundred microliters of the 200 nM CeO2 working solution was added per well to obtain a 10 nM final concentration, incubated for 24 and 48 hours respectively. After incubation, the media was removed and saved in 2.0 mL polypropylene tubes. Then the cells, still attached to the wells, were washed three times with ice cold Ca2+/Mg2+ free PBS. A 500 uL volume of trypsin EDTA was added per well and the plates were returned to the incubator till the cells detached from the plates (3-5 minutes). Then the tripsinized cells were transferred from the wells to a clean eppendorf tube and 500 µL of media was added to the wells to wash any cells remaining in the well. The media was then combined to the tripsinized sample to stop the trypsin reaction. The eppendorf tube containing the cells were spun at 3000 rpm for 5 minutes. The supernatant was removed and the cell pellet was washed three times with 300 µL of Ca2+/Mg2+ free PBS. The cell pellet was suspended in 500 µL of Ca2+/Mg2+ free PBS. A 50uL sample was collected and storage in a 1.5 mL eppendorf at -80°C for protein assay. The obtained pellet sample was stored at -20°C and shipped to the Notre Dame University ICP/MS laboratory for ICP/MS analysis.

ICP/MS results interpretations

Two isotopes of ceria were analyzed, the Ce140 and the Ce142. The ICP/MS results were obtained in parts per million (PPM) and expressed in mg/L and the ceria concentration was normalized according to the protein concentration in the sample.

Protein concentrations determinations

The Pierce BCA protein assay is a colorimetric assay the use bicinchoninic acid to quantify the amount of protein in the sample. The reaction reduces Cu+2 to Cu+1 by protein in an alkaline environment along with the detection of the cuprous cation using a

reagent containing BCA. The purple reaction product absorbs strongly using the 540 nm or 560 nm filter and is linear with increasing protein concentrations between 20 µg/mL and 2000 µg/mL.

Assay Procedure

BCA Working Reagent Preparation

The working reagent was made according to the Pierce kit insert. The working solution was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. Working reagent was made so that there was enough to add 200 µL to each 96-well microplate sample.

BSA Standard Curve

Using the 2.0 mg/mL BSA stock solution, a set of protein standards was made by diluting the BSA into the PBS. An appropriate amount of BSA was added to the PBS so that the desired concentration was achieved. Eight dilutions were made ranging in concentrations between 20 to 2000 µg/mL. Each dilution was mixed well.

96 well microplate procedure

Twenty-five microliters of each sample and the BCA standards were pipette into the appropriate wells in triplicate. For the designated blank samples, 25 µL of PBS was substituted for the BSA standard. Then 200 µL of the working reagent was added to each well, taking care that the samples were mixed well. The microplate was placed on a plate shaker (Janke & Kunkel

GmbH & Co. KG IKA-Labortechnik, Staufen, Germany) for 30 seconds. Then the plate was put in an incubator (Lab-Line Instruments, Inc., Melrose Park, Illinois) at 37 °C for 30 minutes. After incubation, the plate was cooled to room temperature. This took approximately 30 minutes. A template was created that included both the blank and sample wells using the KC4 v2.5 (Biotek instrument, Inc) plate reader software. The plate was read using the BCA Pierce parameters specified above and the data was exported to data analysis software. The protein concentration was expressed in microgram of protein per milliliter of sample (μg/mL).

BCA analysis results

The optical density of the blanks was subtracted from the optical density of the samples to obtain a net optical density. These values were graphed against the protein concentration (μg/mL) contained in the BSA standards.

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 using the Sigma Plot software and a P-value equal to or less than 0.05 was considered statistically significant.

RESULTS

Rat astrocytes exposed to a single dose of 10 Gy ionizing radiation uptake propidium iodine stain 24 and 48 hrs after the radiation insult.

Previous studies showed that dead rat astrocytes, post-exposure to a variety of chemically and mechanically damaging procedures, uptake propidium iodine. The propidium iodine is a fluorescent dye that can be observed under a fluorescent microscope after being taken up by cells in the beginning phase of cell demise. When the cell membrane is compromised, the fluorescent dye enters the cell and the amount of cell demise can be quantified. To determine the deleterious effect of ionizing radiation on rat astrocytes, cells were grown in six well plates to confluency. The plates were then exposed to a single dose of 10 Gy ionizing radiation and were immediately returned to the incubator. At 24 hours after the radiation insult, propidium iodine was added to the cells and they were observed under the fluorescent microscope. Control wells consisted of cells that received a sham radiation dose. Propidium iodine uptake was evaluated by methods described in the Materials and Methods. As shown in figure 1, rat astrocytes exposed to 10 Gy ionizing radiation insult showed increased propidium iodine uptake. Propidium iodine uptake was not observed in the control cells that received the sham radiation.

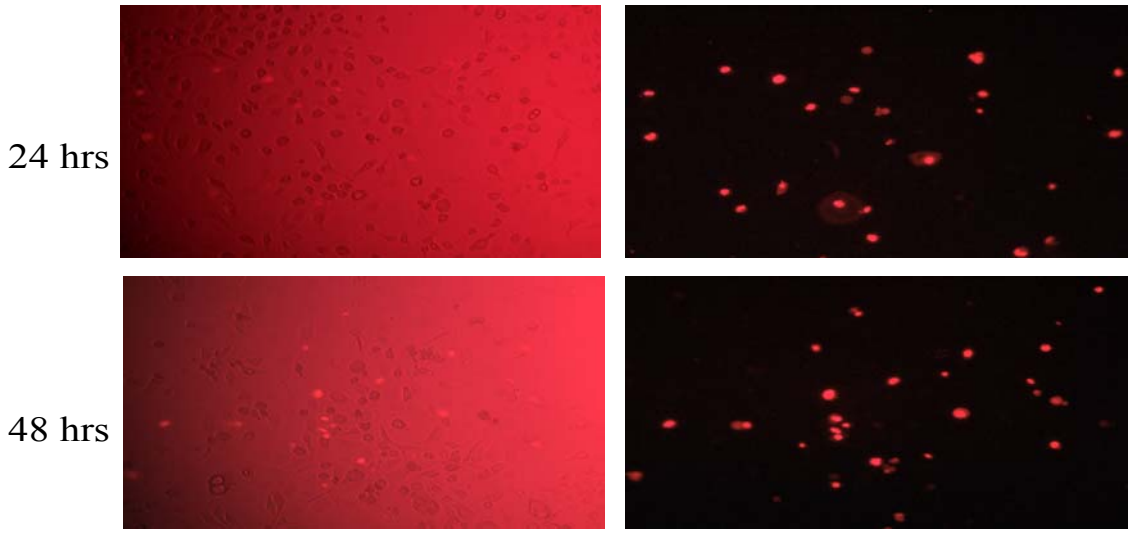

Figure 1. Rat astrocytes exposed to a single dose of 10 Gy ionizing radiation uptake propidium iodine stain 24 and 48 hrs after the radiation insult.

Propidium iodine uptake was observed under a fluorescent microscope 24 and 48 hours after radiation exposure. Control plates that received a sham radiation dose showed only minimal propidium iodine uptake. (Control plates picture not shown).

Rat astrocytes with cerium oxide exposed to 10 Gy ionizing radiation take up minimal propidium iodine stain at 24 and 48 hours after radiation insult

Having established a model of radiation induced cell death, we next examined whether the addition of cerium oxide protect the rat astrocytes from radiation induced cell death. In order to analyze if cerium oxide protects against the deleterious effect of ionizing radiation on rat astrocytes, cells were grown on six well plates to confluency. Cerium oxide was added, to a final concentration of 10 nM, 24 hours prior to radiation. The plates were then exposed to single dose of 10 Gy ionizing radiation and returned to the incubator. At 24 and 48 hours post radiation insult, propidium iodine was added to the cells and observed under the fluorescent microscope. Control cells received cerium oxide solution to a final concentration of 10 nM and were given a sham radiation dose. Propidium iodine uptake was observed and quantified by methods described in the Materials and Methods. As shown in figure 2, propidium iodine uptake was minimal in rat astrocytes pretreated with cerium oxide nanoparticles 24 hours prior to the single dose of 10 Gy ionizing radiation. These results suggest that cerium oxide nanoparticles confer protection against radiation injury. The control plates showed minimal propidium iodine uptake. (Control pictures not shown).

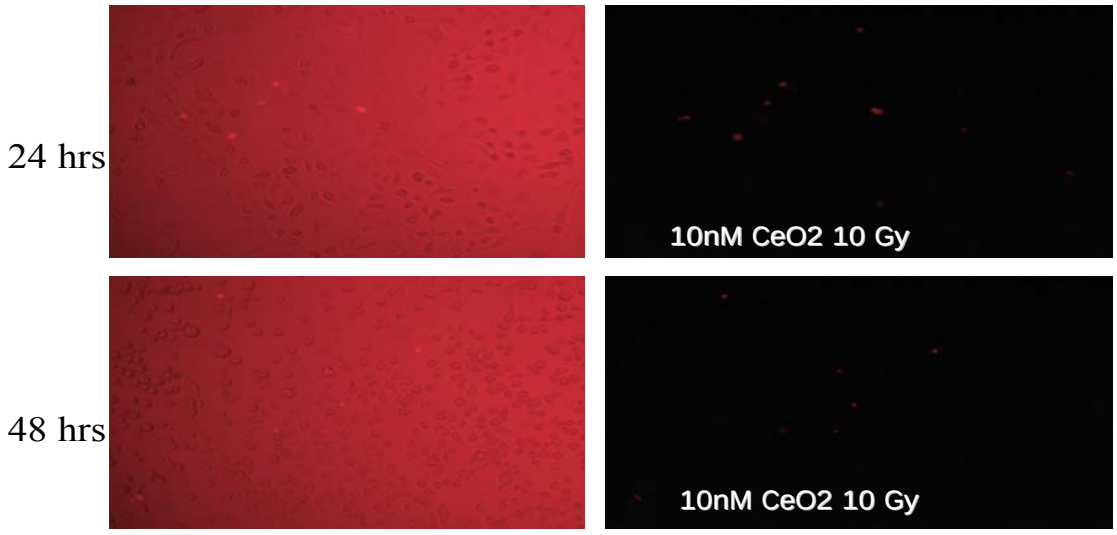

Figure 2. Rat astrocytes with cerium oxide nanoparticles exposed to 10 Gy ionizing radiation at 24 and 48 hrs after radiation insult.

Propidium iodine uptake was observed under a fluorescent microscope 24 and 48 hours after radiation exposure. Control plate that received a sham radiation dose and a final concentration of 10nM cerium oxide nanoparticles showed a minimal propidium iodine uptake as well. (Control plates picture not shown).

Propidium iodine count on rat astrocytes after 10 Gy ionizing radiation

Using this in vitro model we suggested that 10 Gy dose of ionizing radiation is sufficient to induce cell death in rat astrocytes. We counted an average of four propidium iodine positive cells per field at 24 hours after radiation insult and an average of 3 cells per field at 48 hours after radiation insult. When cerium oxide nanoparticles were added, to a final concentration of 10 nanomolar, 24 hours before radiation, we counted two positive propidium iodine positive cells at 24 hours after radiation and 1 propidium iodine positive cell at 48 hours after radiation. The propidium iodine count in cerium oxide treated cells after radiation decreased by two fold at 24 hours after radiation and by almost three fold at 48 hours after radiation. When compared with the control who received the cerium oxide nanoparticles and a sham dose of radiation, we can suggest that the cerium oxide nanoparticles may render protection from ionizing radiation on rat astrocytes. (The background cell count was 25 +/- 3 cells per field).

In a separate experiment cerium oxide nanoparticles was added 5 days prior to radiation. We observed no significant difference between addition of cerium oxide nanoparticles 24 hours before radiation or 5 days before radiation. From figure 3 we can suggest that cells may retain the cerium oxide nanoparticles for up to five days and still rendering the same amount of protection.

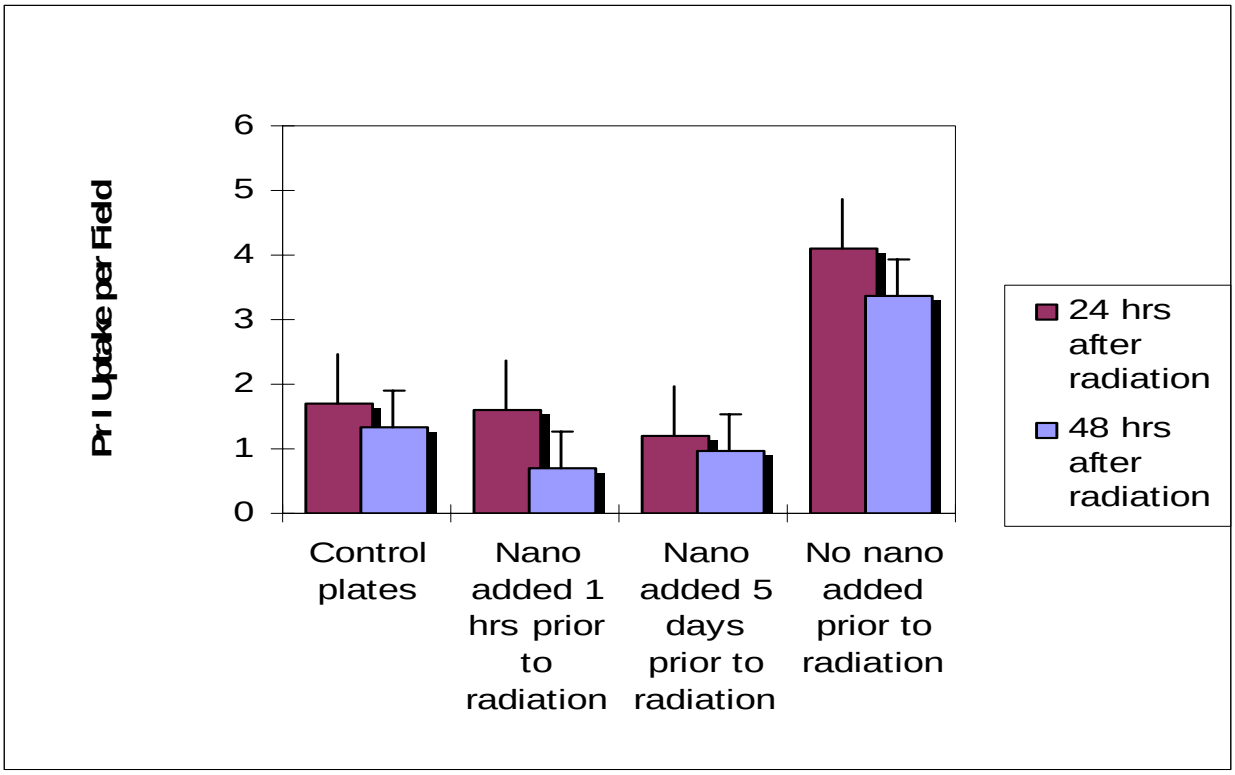

Figure 3. Propidium iodine count on rat astrocytes after 10 Gy ionizing radiation.

Rat astrocytes with a single dose of cerium oxide nanoparticles (10nM) were exposed to 10 Gy ionizing radiation show a minimum amount of propidium iodine stain at 24 and 48 hours after radiation insult. Propidium iodine uptake was observed under a fluorescent microscope and a propidium iodine positive cells per field was counted. Control plate that received a sham radiation dose and a final concentration of 10nM cerium oxide nanoparticles showed a low propidium iodine positive cells per field count as well. Cerium oxide nanoparticles added 5 days prior to radiation showed a similar protection effect. Data are mean +/- S.E. (bars) values of 3 separate experiments performed in triplicate. *$P<0.05$ versus control.

Radiation-induced Cell death of human normal breast cells (CRL8798)

We study the effects of different ionizing radiation dosages in normal breast cells (CRL 8798) at different time interval post radiation. Normal breast cells were plated in 6 well plates. Plates were irradiated at the dose of 0, 1.5, 3 and 10 Gy, returned to a 37ºC incubator and viability measured at 24 and 48 hours by Trypan Blue exclusion assay. It was determined for CRL 8798, that 10 Gy irradiation and assay for viability at 48 hours reflected a greater than LD_{50} dose of ionizing radiation (Figure 4). At 24 hours post radiation, we noticed the percentage of viable cells remained close to the non irradiated control. This can be attributed to the fact that radiation injury has a delay effect on cell death. During the first 24 hours post radiation the cell goes through a cell cycle arrest that permits the intrinsic repair mechanism of the cell to repair any damage associated with the radiation insult. Cell cycle flow cytometry data showed a G1 arrest at 24 hours after radiation insult (Data not shown).

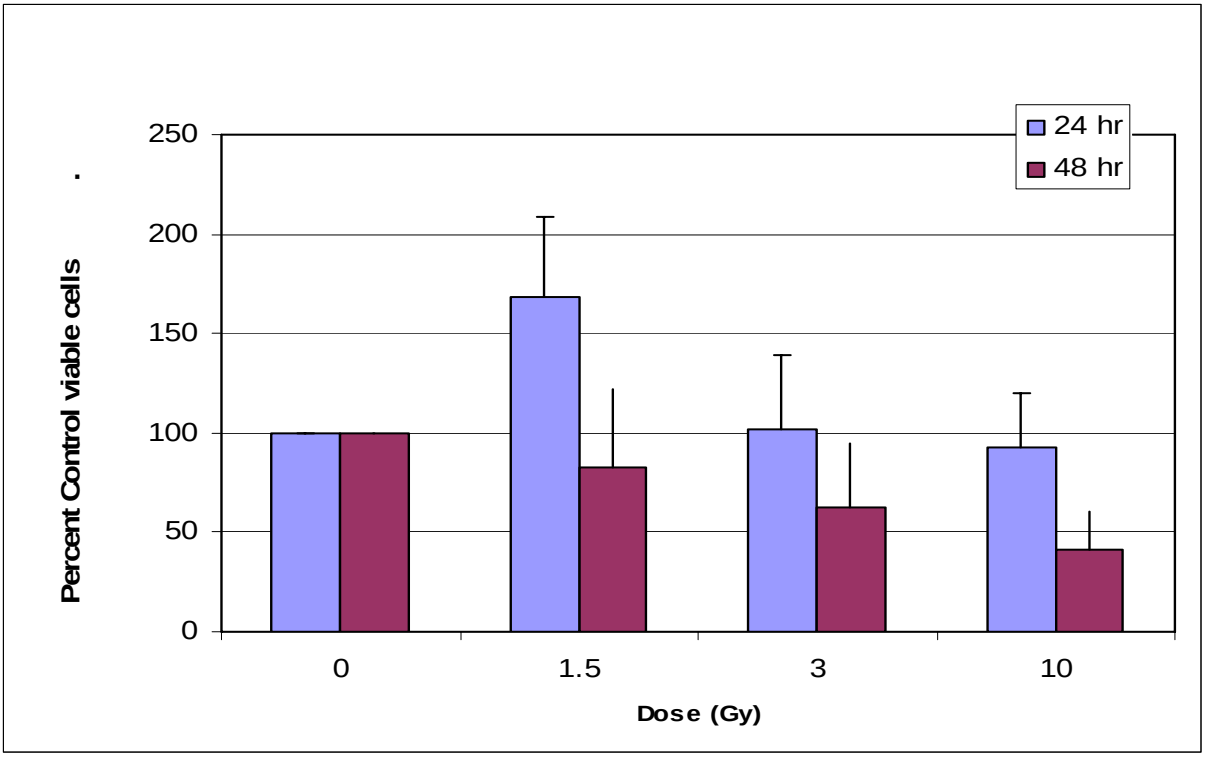

Figure 4. Radiation-induced cell death of human normal breast cells (CRL8798)

Cells were grown to confluency in 96 wells plates. Then plates were exposed to different dosages of ionizing radiation using a Varian Linear Accelerator. Cell viability was assessed by trypan blue exclusion at 24 and 48 hours after radiation exposure. Control plates received a sham dose of radiation. All results are expressed in percent of the control.

Radiation-induced cell death of human tumor breast cells (MCF-7)

We study the effects of different ionizing radiation dosages in tumor breast cells (MCF-7) at different time interval post radiation. Tumor breast cell lines were plated in 6 well plates. Plates were irradiated at the dose of 0, 1.5, 3 and 10 Gy, returned to a 37ºC incubator and viability measured at 24 and 48 hours by Trypan Blue exclusion assay. It was determined for MCF-7, that 10 Gy irradiation and assay for viability at 48 hours reflected a greater than LD_{50} dose of ionizing radiation (Figure 5). At 24 hours post radiation, we noticed that a lower radiation dosages of 1.5 and 3.0 Gy the percentage of viable cells remained close to the non irradiated control. This similar effect to the normal cells can be attributed to the fact that radiation injury has a delayed effect on cell death. During the first 24 hours post radiation the cell goes through a cell cycle arrest that permits the intrinsic repair mechanism of the cell to repair any damage associated with the radiation insult. We obtained cell cycle flow cytometric data showed a G1 arrest at 24 hours after radiation insult (Data not shown).

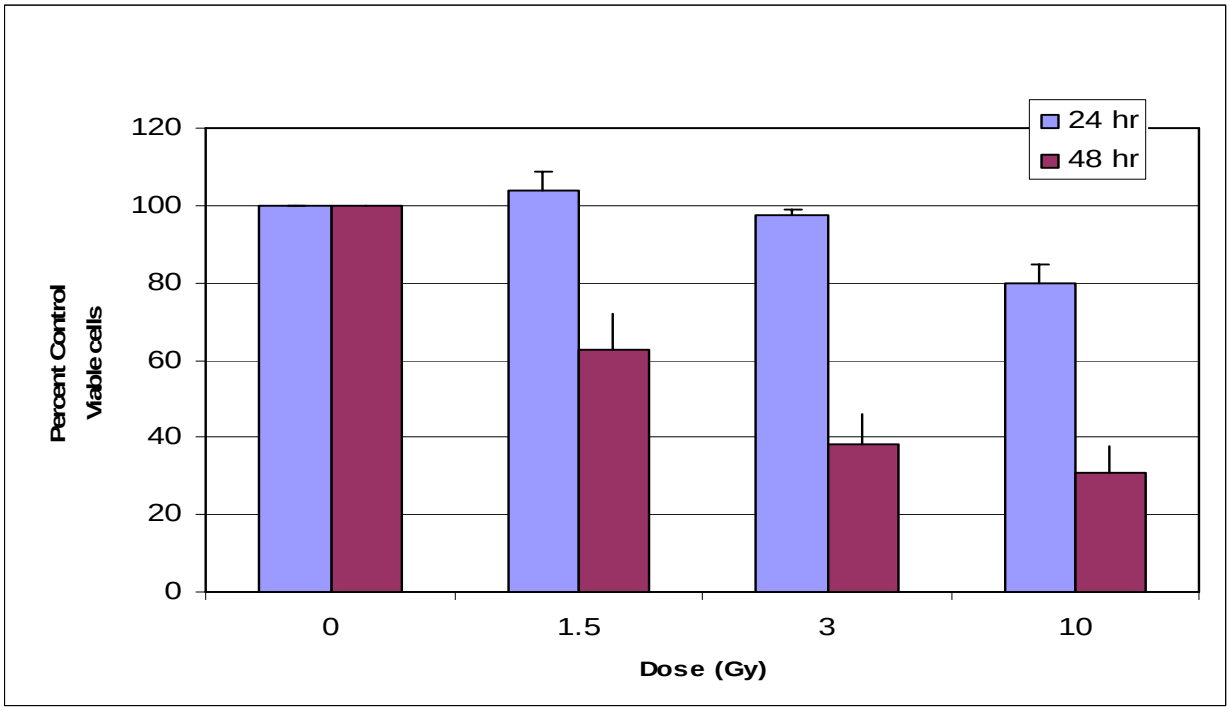

Figure 5. Radiation-induced cell death of human tumor breast cells (MCF-7)

Cells were grown to confluency in 96 wells plates. Then plates were exposed to different dosages of ionizing radiation using a Varian Linear Accelerator. Cell viability was assessed by trypan blue exclusion at 24 and 48 hours after radiation exposure. Control plates received a sham dose of radiation. All results are expressed in percent of the control.

Cerium oxide nanoparticles effects on cell viability of human normal breast cells CRL8798 following a 10 Gy dose of ionizing radiation at 24 hours.

Normal breast cells were plated in 96 well plates and cell proliferation was evaluated using an MTT assay. After exposing the cells to a single dose of 10 Gy ionizing radiation, an almost 25% decrease in cell proliferation was observed 24 hours after the radiation insult. When the cells were treated with 10 nM CeO2 nanoparticles 24 hours prior to radiation, we observed similar cell proliferation to the control cells (Figure 6). This conservation of cell proliferation, after exposure of nanoceria treated cells to ionizing radiation, suggests that the cerium oxide nanoparticles may be rendering protection against the deleterious effects of radiation. The pretreatment with nanoceria did not demonstrate any significant effects on normal breast cells that were not irradiated. This observation support previous results showing that cerium oxide nanoparticles have no cytotoxic effects on normal breast cells 48 hours after addition of cerium oxide to the cultures. (Data not shown).

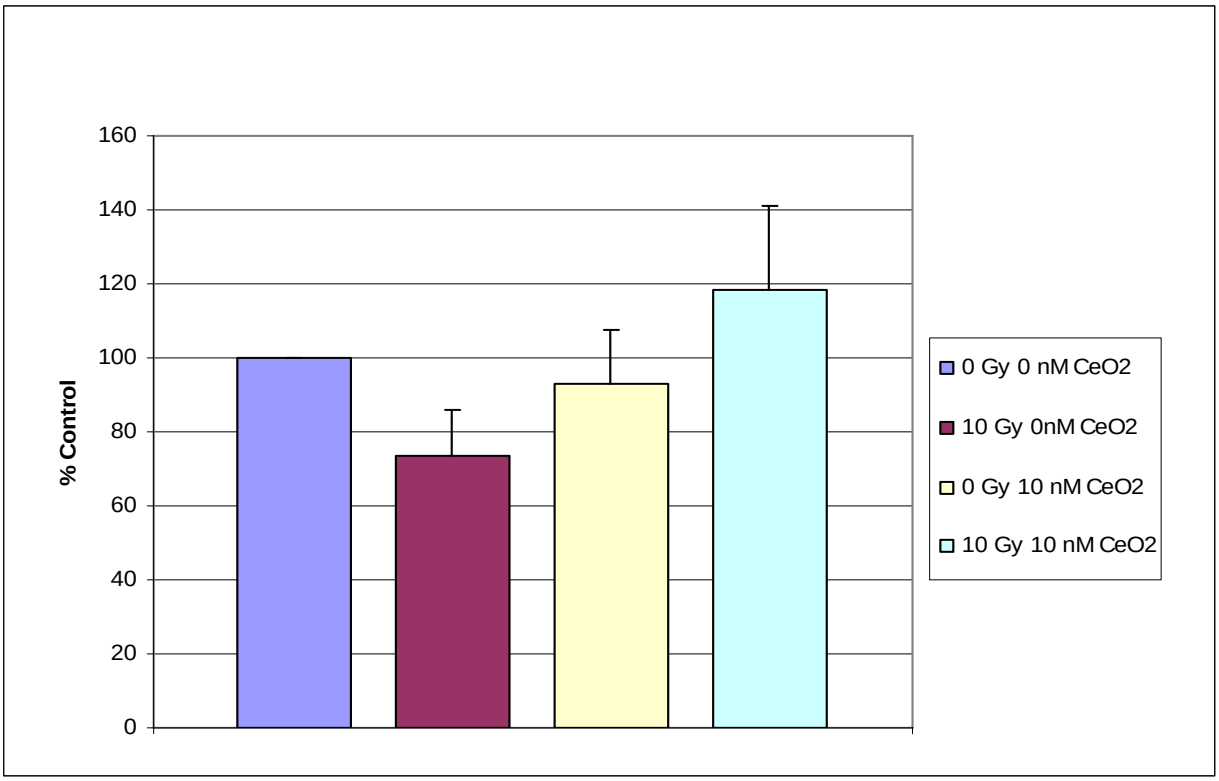

Figure 6. Cerium oxide nanoparticles effects on cell viability of human normal breast cells CRL8798 following a 10 Gy irradiation dose at 24 hours.

Five thousands cells per well were seeded and grown to confluency in 96 wells plates. Then plates were exposed to a single 10 Gy dose of ionizing radiation using a Varian Linear Accelerator. Cell viability was assessed by MTT assay at 24 hours after radiation exposure. Control plates received a sham dose of radiation. All results are expressed in percent of the control.

Cerium oxide nanoparticles effects on cell viability of human tumor breast cells MCF-7 following a 10 Gy dose of ionizing radiation at 24 hours.

Tumor breast cells were plated in 96 well plates and cell proliferation was evaluated by MTT assay. When cell plates were irradiated with a single dose of 10 Gy, MCF-7 cells were killed at level of 20-30% at 24 hours after radiation insult. When pretreated with 10 nM cerium oxide nanoparticles 24 hours prior to irradiation, MCF-7 cells showed no statistically significant difference in cell proliferation between cells that were not treated (Figure 7). The pretreatment did not demonstrate any significant effect on the cell proliferation of tumor breast cells that were not irradiated. This observation support previous results that show that cerium oxide nanoparticles has no cytotoxic effects on tumor breast cells at 48 hours after treatment (Data not shown).

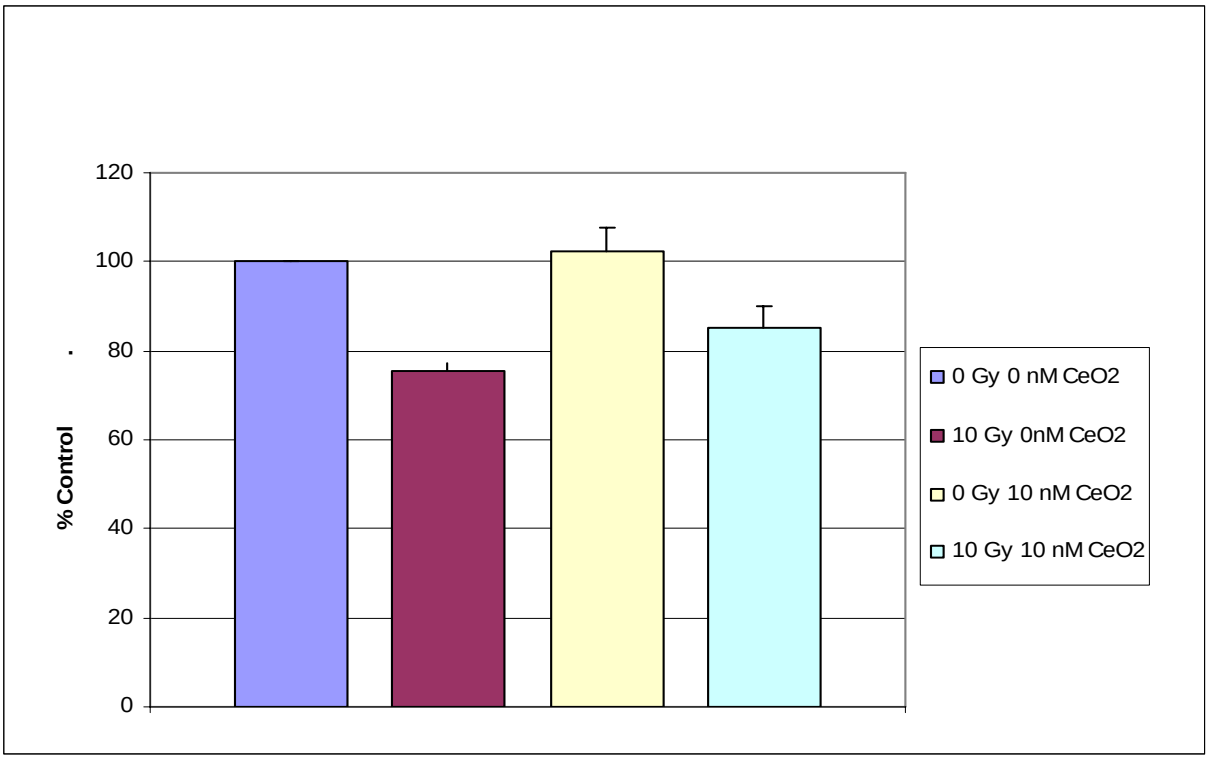

Figure 7. Cerium oxide nanoparticles effects on cell viability of human tumor breast cells MCF-7 following a 10 Gy ionizing radiation dose at 24 hours.

Five thousands cells per well were seeded and grown to confluency in 96 wells plates. Then plates were exposed to a single 10 Gy dose of ionizing radiation using a Varian Linear Accelerator. Cell viability was assessed by MTT assay at 24 hours after radiation exposure. Control plates received a sham dose of radiation. All results are expressed in percent of the control.

Cerium oxide nanoparticles effects on cell viability of human normal breast cells CRL8798 treated with a 10 Gy dose of ionizing radiation at 48 hours

Normal breast cells were plated in 96 well plates and cell proliferation was evaluated by MTT assay. After exposing the cells to a single dose of 10 Gy ionizing radiation, an almost 50% decrease of cell proliferation as compared to the non irradiated control was observed 48 hours after radiation insult. When the cells were pretreated with 10 nM CeO2 nanoparticles 24 hours prior to radiation, almost no change in cell proliferation was observed as compared to the non-irradiated control after 48 hours (Figure 8). This conservation of cell proliferation, after exposure of ionizing radiation to nanoceria treated cells, suggests that the cerium oxide nanoparticles may be rendering close to 100% protection against the deleterious effect of radiation. The pretreatment did not demonstrate any significant effect on normal breast cells that were not irradiated. This observation support previous results that show that cerium oxide nanoparticles have no cytotoxic effects on normal breast cells at 72 hrs after addition the cerium oxide nanoparticles in the cultures. (Data not shown).

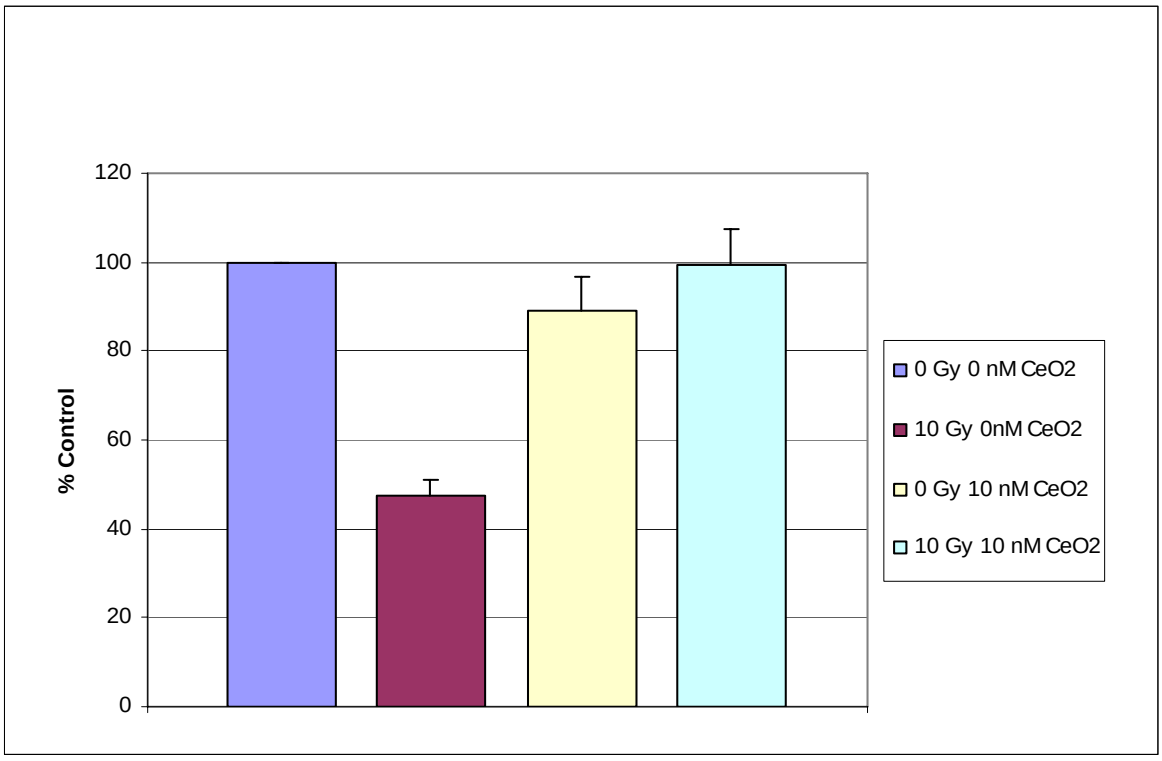

Figure 8. Cerium oxide nanoparticles effects on cell viability of human normal breast cells CRL8798 following a 10 Gy ionizing radiation dose at 48 hours.

Five thousands cells per well were seeded and grown to confluency in 96 wells plates. Then plates were exposed to a single 10 Gy dose of ionizing radiation using a Varian Linear Accelerator. Cell viability was assessed by MTT assay at 48 hours after radiation exposure. Control plates received a sham dose of radiation. All results are expressed in percent of the control.

Cerium oxide nanoparticles effects on cell viability of human tumor breast cells MCF-7 treated with a 10 Gy dose of ionizing radiation at 48 hours.

Tumor breast cells were plated in 96 well plates and cell proliferation was evaluated by MTT assay. When cell plates were irradiated with a single dose of 10 Gy, MCF-7 were killed at level of 20-30% from non irradiated control at 48 hours after radiation insult. When pretreated with 10 nM cerium oxide nanoparticles 24 hours prior to irradiation, MCF-7 cells showed no statistically significant difference in cell proliferation between cells that were not treated, from the irradiated non treated control. (See Figure 9). We also observed a similar non cytotoxic effect from cerium oxide nanoparticles on MCF-7 cells. The pretreatment did not demonstrate any significant effect on tumor breast cells the not irradiated control. This observation support previous results that show that cerium oxide nanoparticles has no cytotoxic effects on tumor breast cells at 72 hours after treatment (Data not shown).

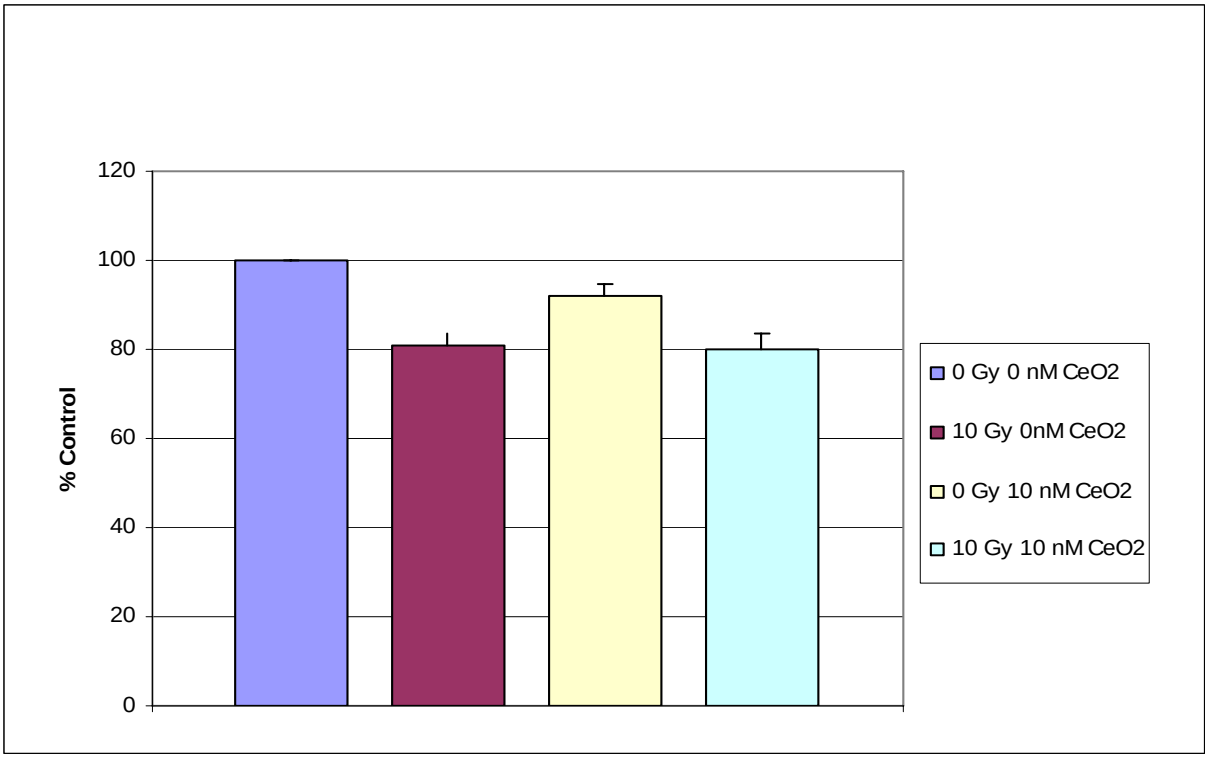

Figure 9. Cerium oxide nanoparticles effects on cell viability of human tumor breast cells MCF-7 following a 10 Gy irradiation dose at 48 hours.

Five thousands cells per well were seeded and grown to confluency in 96 wells plates. Then plates were exposed to a single 10 Gy dose of ionizing radiation using a Varian Linear Accelerator. Cell viability was assessed by MTT assay at 48 hours after radiation exposure. Control plates received a sham dose of radiation. All results are expressed in percent of the control.

Cerium oxide nanoparticles effects on apoptotic cell death of human breast cells following a 10 Gy irradiation dose at 48 hours.

Subsequent experiments were conducted to look at the protective effect of cerium oxide nanoparticles on radiation-induced apoptotic cell death. It is well known that ionizing radiation (x-ray, gamma rays) can induce damage to the DNA by directly attacking its sugar back bone and causing double strand breaks or "nicks" in the DNA molecule. We used the TUNEL assay method to detect this nicks in cells that were injured by the delivered dose of ionizing radiation and subsequently went apoptotic. To study this, the cells were grown on chamber slides and pretreated to a final concentration of 10 nM cerium oxide nanoparticles for 24 hours. After the 24 hours incubation slides were irradiated with a single dose of 10 Gy and incubated at 37ºC for 48 hours. Control consisted of cells irradiated and not treated with cerium oxide and cells treated with cerium oxide and not irradiated. Cell death was measured by TUNEL staining for both the normal CRL8798 cells and the tumor MCF-7 cells. As seen previously with the Trypan Blue exclusion and the MTT cell viability assays, cells can be injured following radiation exposure, and induced to undergo apoptosis. When pretreating the cells, 24 hours prior to radiation, with 10 nM cerium oxide nanoparticles (final concentration), CRL8798 cells showed minimal TUNEL positive cells suggesting almost 100% protection from radiation-induced apoptosis whereas MCF-7 cells showed TUNEL positive cells on both the pretreated and the untreated cells suggesting no significant protection by the CeO2 nanoparticles (Figure 10).

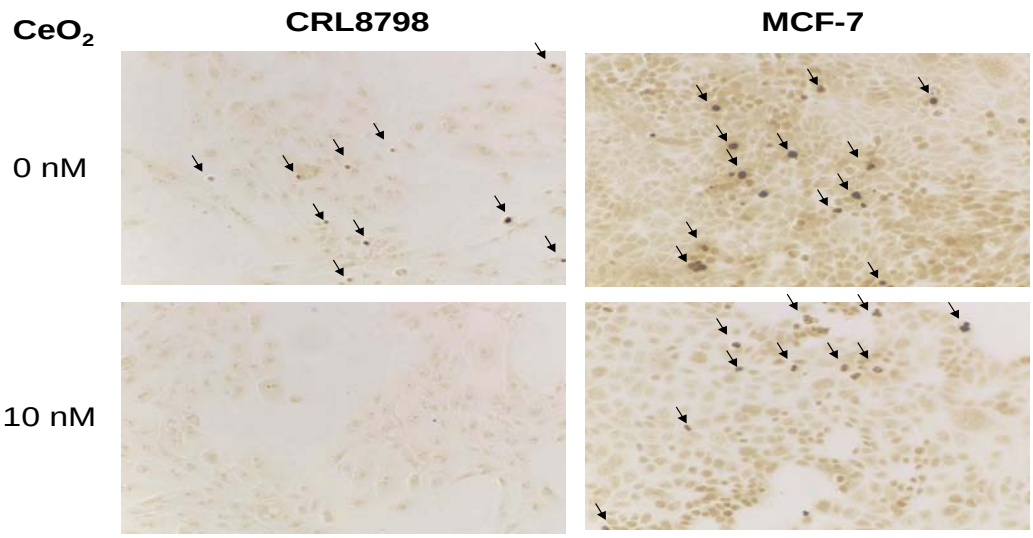

Figure 10. TUNEL staining of breast cells at 48 hours following 10 Gy irradiation and protection by cerium oxide nanoparticles.

Cell death was determined by TUNEL cell staining of CRL8798 and MCF-7 cells following 10 Gy irradiation with or without a cerium oxide nanoparticles (final concentration of 10 nM) at 48 hours after radiation. Fifty thousands cells per well were seeded and grown to confluency in chamber slides. Then the individual slides were exposed to a single 10 Gy dose of ionizing radiation using a Varian Linear Accelerator. Cell death was assessed at 48 hours after radiation exposure. Control slides received a sham dose of radiation (Data not shown). Arrows point to TUNEL positive or apoptotic cells.

Cerium oxide nanoparticles protects CRL 8798 cells from radiation induced apoptosis.

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 cerium oxide nanoparticles to a final concentration of 10 nM for 24 hours. Then, slides were irradiated with a single 10 Gy and incubated at 37ºC for 24 hours. Cell death, for adherent and non-adherent cells, was measured by TUNEL staining for normal cells, CRL 8798. (As seen with the Trypan Blue exclusion and the MTT cell viability assays, CRL8798 can be induced to undergo apoptosis following radiation exposure). We noted that after radiation exposure an average of about 5% of the cells stained positive for TUNEL. This results support previous results reported by Bloomfield et. al. 2003 where they reported an ionizing radiation minimal cell kill of a 6% apoptotic cells in normal prostate cells exposed to a single 10Gy radiation dose. When CRL 8798 cells were pretreated for 24 hours with the nanoparticles, and irradiated we counted 1% TUNEL positive cells. These results suggest that the cerium oxide nanoparticles may be rendering a 100% protection from radiation-induced apoptosis in normal breast cells (Figure 10). We also noted that the addition of cerium oxide nanoparticles alone (no radiation control) decreased the number of TUNEL positive cells (1%) when compared with the control (no cerium oxide, no radiation) in which we counted a 2% TUNEL positive cells. These may suggest that the cerium oxide nanoparticles may indirectly protect the cells from radiation by a mechanism other than acting as a free radical scavenger themselves. Other studies should be conducted to determined how cerium oxide affect the gene expression in normal cells and what genes get upregulated and/or downregulated following the addition of cerium oxide. It may be correct to

speculate that the presence of ceria may help to prepare or prime the cell for any action that can be deleterious to their biological environment.

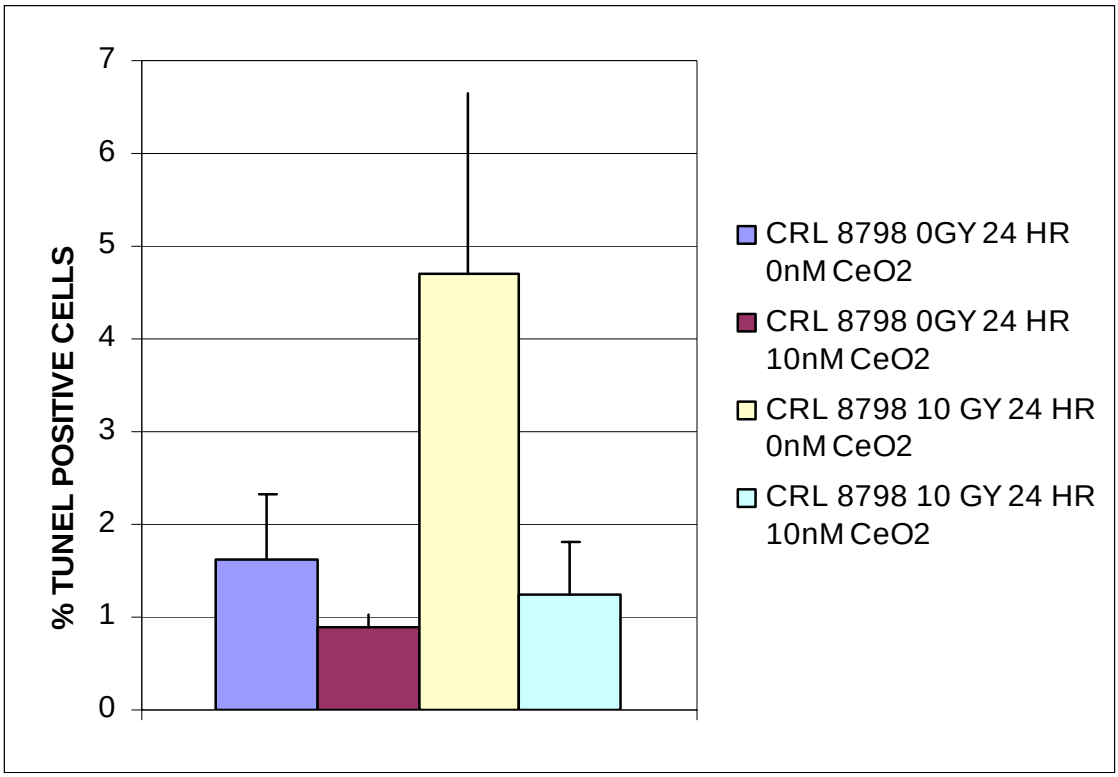

Figure 11. Cerium oxide nanoparticles protect CRL 8798 cells from radiation induced apoptosis.

To determine cell apoptosis in normal cells, 50,000 cells were seeded in a chamber slide and grown to confluency. Then, cells were treated with cerium oxide nanoparticles to a final concentration of 10 nM 24 hours prior to radiation. After a 24 hours incubation the slides were exposed to a single 10 Gy dose of ionizing radiation and return to the incubator at 37C and 5% CO2. At 24 after the radiation insult, cells were stained for TUNEL according to the manufacturer instructions. TUNEL positive cells were observed under a light microscope at 40X and 10 random field were counted in a double blinded manner. The percentage of TUNEL positive cells was calculated for every slide using the following formula: (TUNEL positive / Total cell in a field)*100.

Cerium oxide nanoparticles protect CRL 8798 cells from radiation induced apoptosis at 48 hours.

To look at the protective effect of cerium oxide nanoparticles on radiation-induced apoptotic cell death 48 hours after the radiation insult, the normal breast cells were grown on chamber slides and pretreated with cerium oxide nanoparticles to a final concentration of 10 nM and incubated for 24 hours. Then, slides were irradiated with a single 10 Gy and incubated at 37ºC for 48 hours. Cell death, for adherent and non-adherent cells, was measured by TUNEL staining for normal cells, CRL 8798. We noted that after radiation exposure an average of about 4% of the cells stained positive for TUNEL stain. We can see that radiation can induce cell death 48 hours after the lethal exposure. When CRL 8798 cells were pretreated for 24 hours with the nanoparticles, and irradiated we counted 1% TUNEL positive cells or a 3-fold reduction from its counterpart control (irradiated cells not treated with the nanoparticles). These results are mimicking the results observed in the MTT cell proliferation experiments, both suggest that the cerium oxide nanoparticles may be rendering an almost 100% protection from radiation-induced apoptosis in normal breast cells (Figure 11). At 48 hours (72 hours after pretreatment with cerium oxide) we also noted that the addition of cerium oxide nanoparticles alone (no radiation control) did not change significantly the number of TUNEL positive cells (2%) when compared with the control (no cerium oxide, no radiation) which we found to be 2% TUNEL positive cells. These results again corroborates that the presence of the nanoparticles alone are not detrimental to the normal cells.

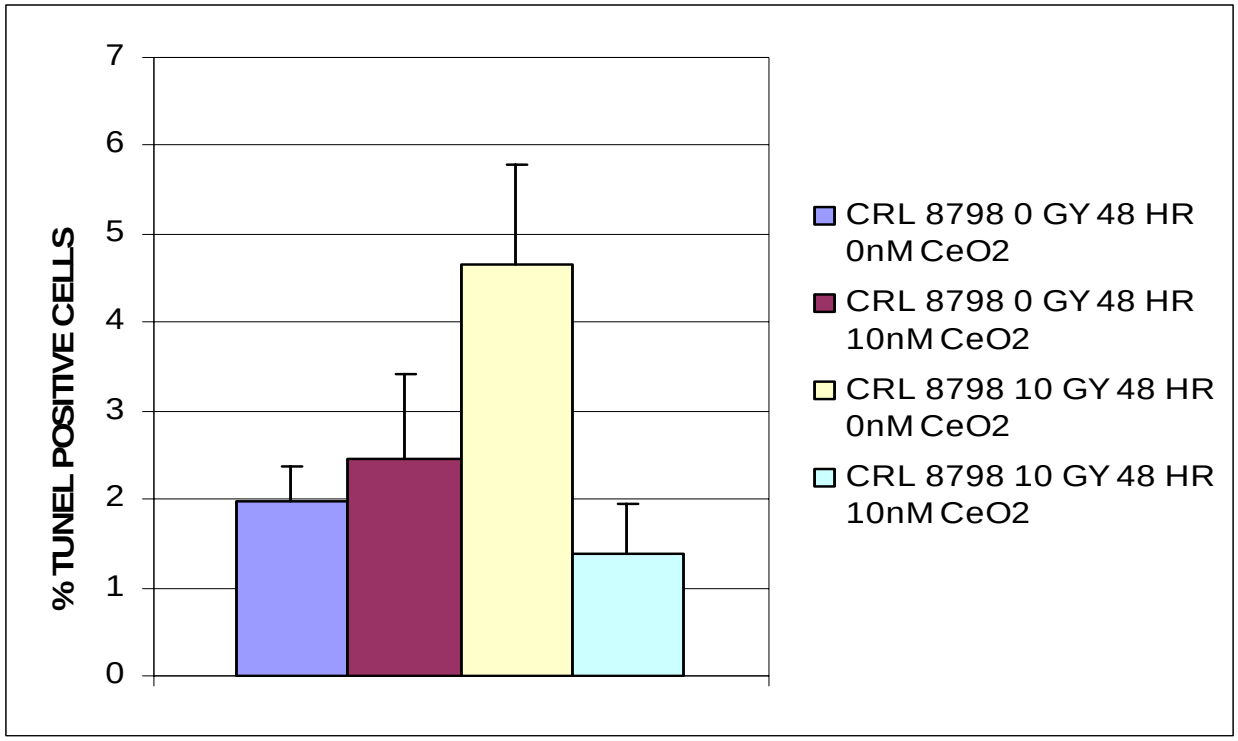

Figure 12. Cerium oxide nanoparticles protect CRL 8798 cells from radiation induced apoptosis at 48 hours.

To determine cell apoptosis in normal cells, 50,000 cells were seeded in a chamber slide and grown to confluency. Then, cells were treated with cerium oxide nanoparticles to a final concentration of 10 nM. After a 24 hour incubation with the nanoparticles, the slides were exposed to a single 10 Gy dose of ionizing radiation and return to the incubator at 37C and 5% CO2. At 48 after the radiation insult, cells were stained for TUNEL according to the manufacturer instructions. TUNEL positive cells were observed under a light microscope at 40X and 10 random field were counted in a double blinded manner. The percentage of TUNEL positive cells was calculated for every slide using the following formula: (TUNEL positive / Total cell in a field)*100.

Cerium oxide nanoparticles provide no protection on breast tumor cells from radiation induced apoptosis 24 hours.

To look at the protective effect of cerium oxide nanoparticles on radiation-induced apoptotic cell death in tumor cells, the cells were grown on chamber slides and pretreated with cerium oxide nanoparticles to a final concentration of 10 nM for 24 hours. Then, slides were irradiated with a single 10 Gy and incubated at 37ºC for 24 hours. Cell death, for adherent and non-adherent cells, was evaluated by TUNEL staining. We noted that after radiation exposure an average of about 7% of the cells stained positive for TUNEL. When MCF-7 cells were pretreated for 24 hours with the nanoparticles, and irradiated we counted a 8% TUNEL positive cells. Theses results suggest that the cerium oxide nanoparticles may not be rendering any protection from radiation-induced apoptosis in tumor breast cells (Figure 12). We also noted that the addition of cerium oxide nanoparticles alone (no radiation control) unlike in the normal cells, did not change significantly the number of TUNEL positive cells (5%) when compared with the control (no cerium oxide, no radiation) which we found a 5% TUNEL positive cells.

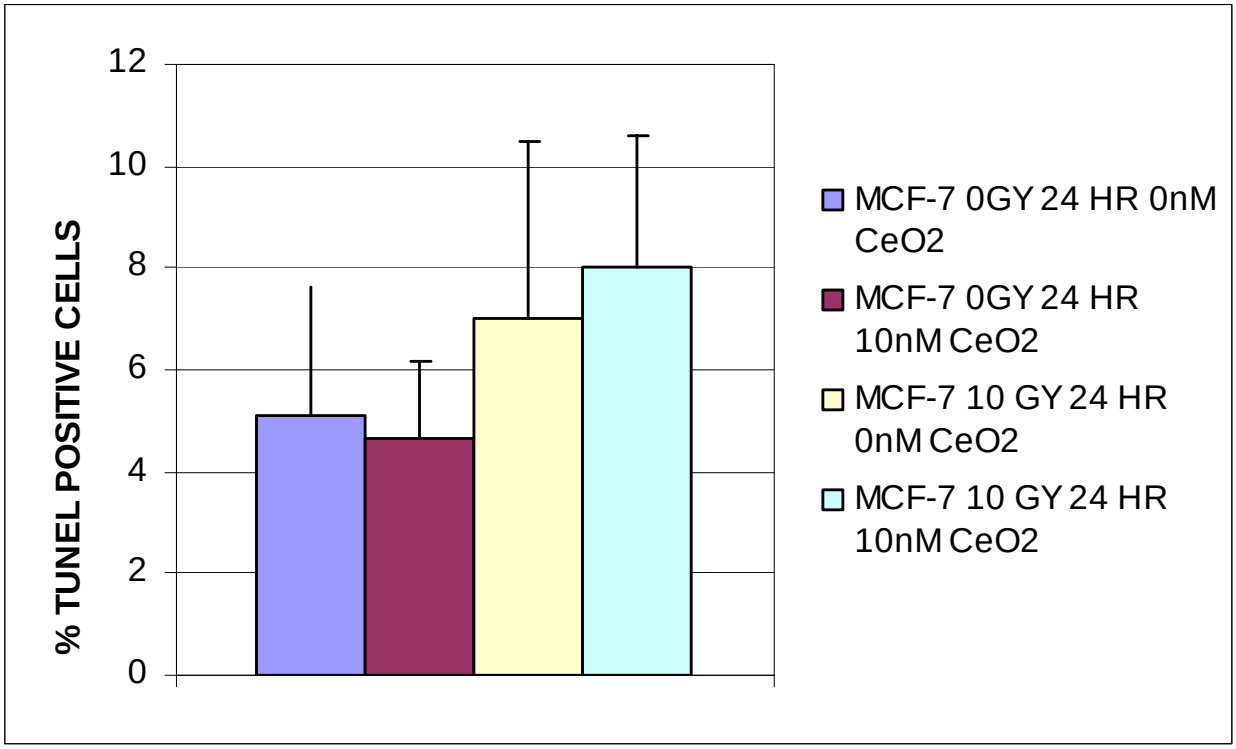

Figure 13. Cerium oxide nanoparticles protection on MCF-7 tumor breast cells from radiation induced apoptosis at 24 hours.

To determine cell apoptosis in normal cells, 50,000 cells were seeded in a chamber slide and grown to confluency. Then, cells were treated with cerium oxide nanoparticles to a final concentration of 10 nM 24 hours prior to radiation. Then, the slides were exposed to a single 10 Gy dose of ionizing radiation and return to the incubator at 37C and 5% CO2. At 24 after the radiation insult, cells were stained for TUNEL according to the manufacturer instructions. Cell positive cells were observed under a light microscope at 40X and 10 random fields were counted in a double blinded manner. The percentage of TUNEL positive cells was calculated for every slide using the following formula: (TUNEL positive / Total cell in a field)*100.

Cerium oxide nanoparticles provide no protection on breast tumor cells from radiation induced apoptosis 48 hours.

To look at the protective effect of cerium oxide nanoparticles on radiation-induced apoptotic cell death in tumor cells, the cells were grown on chamber slides and pretreated with cerium oxide nanoparticles to a final concentration of 10 nM for 24 hours. Then, slides were irradiated with a single 10 Gy and incubated at 37ºC for 48 hours. Cell death, for adherent and non-adherent cells, was evaluated by TUNEL staining. We noted that after radiation exposure an average of about 7% of the cells stained positive for TUNEL. When MCF-7 cells were pretreated for 24 hours with the nanoparticles, and irradiated we counted 8% TUNEL positive cells. These results suggest that the cerium oxide nanoparticles may not be rendering any protection from radiation-induced apoptosis in tumor breast cells (Figure 13). Interestingly, we noted a 1% increase in TUNEL positive stain on cells that were pretreated with the cerium oxide nanoparticles and irradiated. Even thought the difference is of only 1% is need to be address that there a possibility that, in the case of the tumor cells, the nanoparticles may be enhancing the deleterious effect of ionizing radiation. Same as the 24 hours time course, we also noted that the addition of cerium oxide nanoparticles alone (no radiation control) unlike in the normal cells, did not change significantly the number of TUNEL positive cells (4%) when compared with the control (no cerium oxide, no radiation) which we found to be 4% TUNEL positive cells. These suggest that the cerium oxide nanoparticles are not protecting the tumor cells from radiation-induced cell death but in fact might possibly be enhancing the radiation effects.

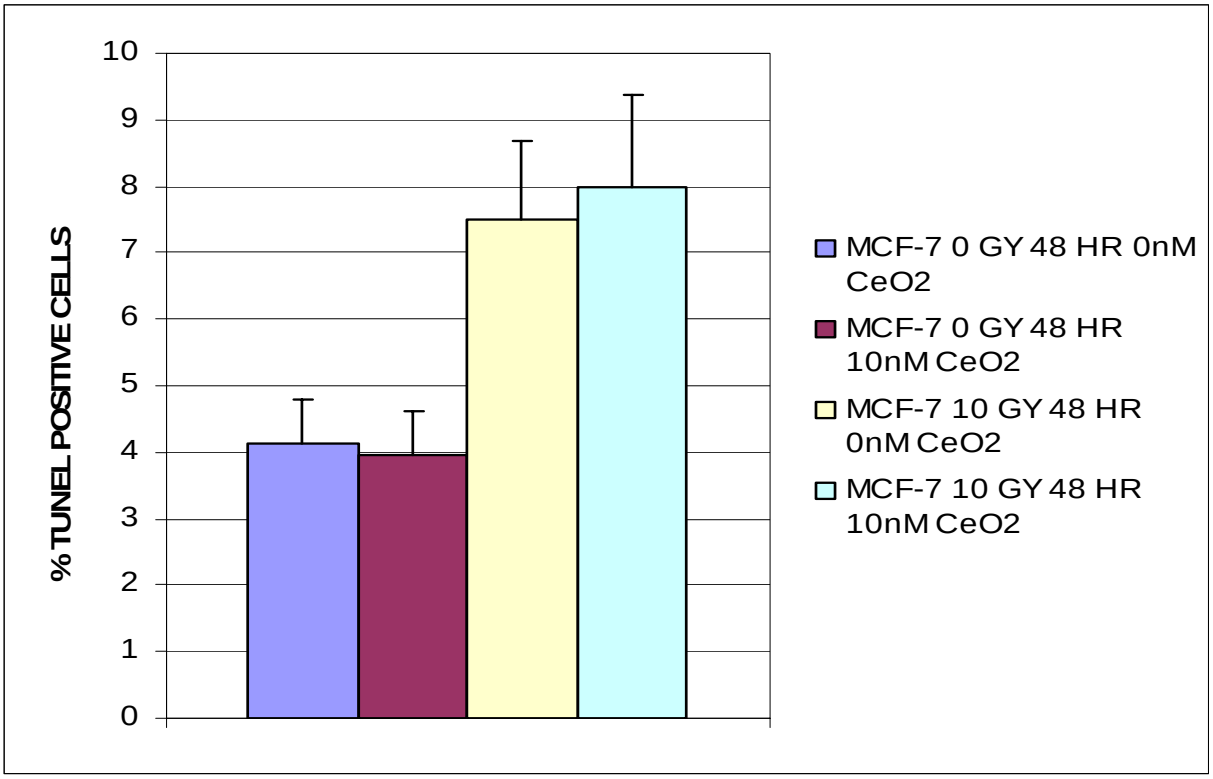

Figure 14. Cerium oxide nanoparticles protective effects on MCF-7 tumor breast cells from radiation induced apoptosis at 48 hours.

To determine cell apoptosis in normal cells, 50,000 cells were seeded in a chamber slide and grown to confluency. Then, cells were treated with cerium oxide nanoparticles to a final concentration of 10 nM at 24 hours prior to radiation. Then, the slides were exposed to a single 10 Gy dose of ionizing radiation and return to the incubator at 37C and 5% CO2. At 48 after the radiation insult, cells were stained for TUNEL according to the manufacturer instructions. TUNEL positive cells were observed under a light microscope at 40X and 10 random field were counted in a double blinded manner. The percentage of TUNEL positive cells was calculated for every slide using the following formula: (TUNEL positive / Total cell in a field)*100.

Protective effect of cerium oxide nanoparticles: Comparisons between normal breast cells (CRL 8798) and breast tumor cells (MCF-7) 24 hours after a 10 Gy single dose of ionizing radiation.

To summarize the results obtained from the TUNEL assays, we compared the percentage increased of TUNEL positive cells between the normal breast cells and the breast tumor cell lines. The percent from control was calculated to compare the effects of ceria nanoparticles in both cell lines. In figure 15 we can see that 24 hours after the radiation insult, tumor cells showed an increase in apoptotic nuclei by 1-fold on both the ceria pretreatment and without the nanoceria pretreatment. We could not observe a significant difference between both conditions. These suggest that the nanoceria pretreatment has not effect on the breast tumor cell line at 24 hours after radiation.

On other hand, the percentage of increased positive TUNEL cells in the normal cell line was relatively different to its tumor counterpart. We observed a 3-fold increase in TUNEL positive cells after radiation in untreated normal cells and a 0.25-fold increase on the cells pretreated with cerium oxide nanoparticles. A protective effect can be clearly observed in normal cells at 24 hours after the radiation insult.

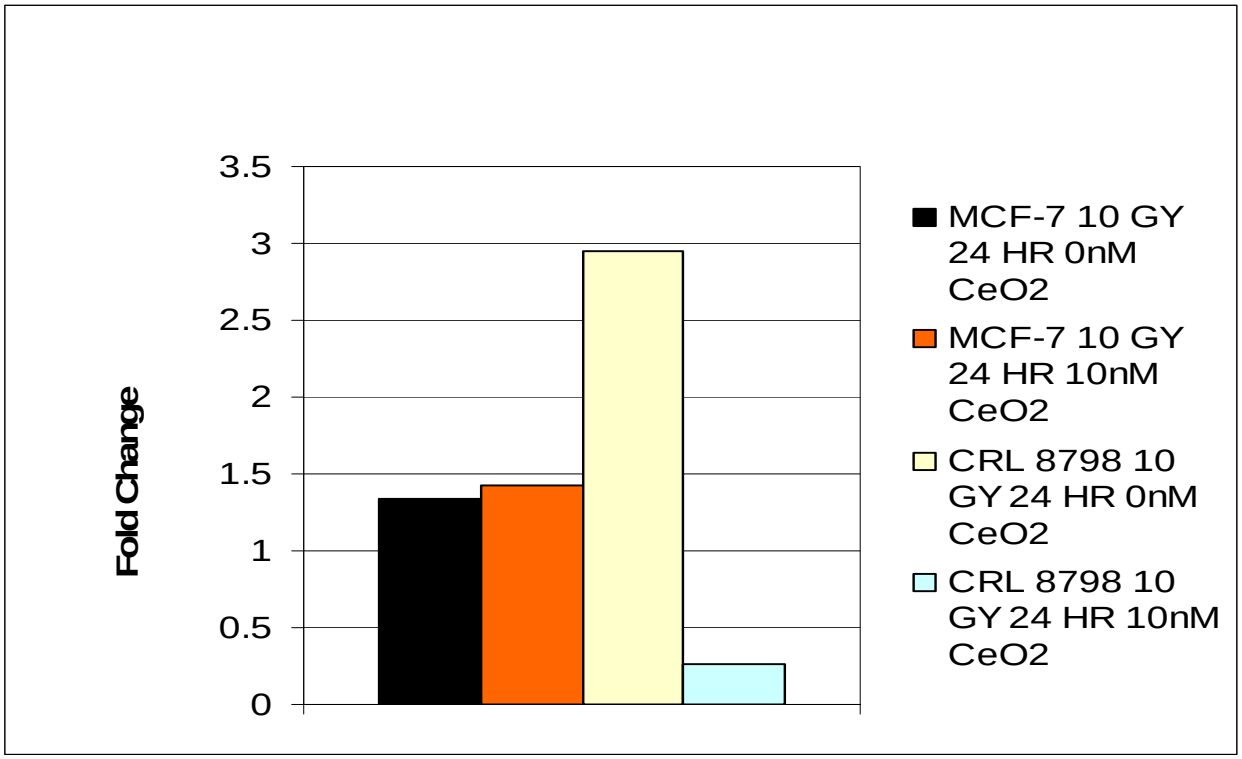

Figure 15. Protective effect of cerium oxide nanoparticles at 24 hours.

A comparison between normal breast cells (CRL 8798) and breast tumor cells (MCF-7) 24 hours after ionizing radiation. The percentage increase of TUNEL positive cells after a 10 Gy single dose of ionizing radiation was evaluated to compare the effects of the cerium oxide nanoparticles in normal and tumor breast cells. We observed a 1- fold increase in TUNEL positive cells on both conditions (treated and untreated) in tumor cell lines. And we observed a 3-fold increased in the untreated normal breast cell line and a 0.25 fold increase in the treated breast normal cell line.

Protective effect of cerium oxide nanoparticles: Comparisons between normal breast cells (CRL 8798) and breast tumor cells (MCF-7) 48 hours after ionizing radiation.

To summarize the results obtained from the TUNEL assays at 48 hours after the radiation insult, we compared the percent of TUNEL positive cells between the normal breast cells and the breast tumor cell lines. The percent from control was calculated to compare the effects of nanoceria in both cell lines. In figure 17, we observed that 48 hours after the radiation insult, tumor cells showed a 2-fold increase in apoptotic nuclei in cells without nanoparticles pretreatment opposed to only a 1-fold increase in the cells that were pretreated with the nanoparticles. We observed that in the presence of nanoparticles, tumor cell lines show a lower percentage of TUNEL positive cells than untreated cells. These results show a potential protective effect of the nanoparticles on cell death in the tumor cell lines at 48 hours post-radiation. Overall we can suggest that the nanoparticles pretreatment has no effect on the breast tumor cell lines.

On other hand, the results observed in the normal cell line were more dramatic than those seen in the tumor cells. We observed in the 48 hour time course, a 2-fold increase in TUNEL positive cells after radiation in untreated normal cells and a 0.3-fold increase in the pretreated cells, which is an almost 2-fold difference between the two conditions. A protective effect can clearly be observed in the normal breast cells at 48 hours after the radiation insult.

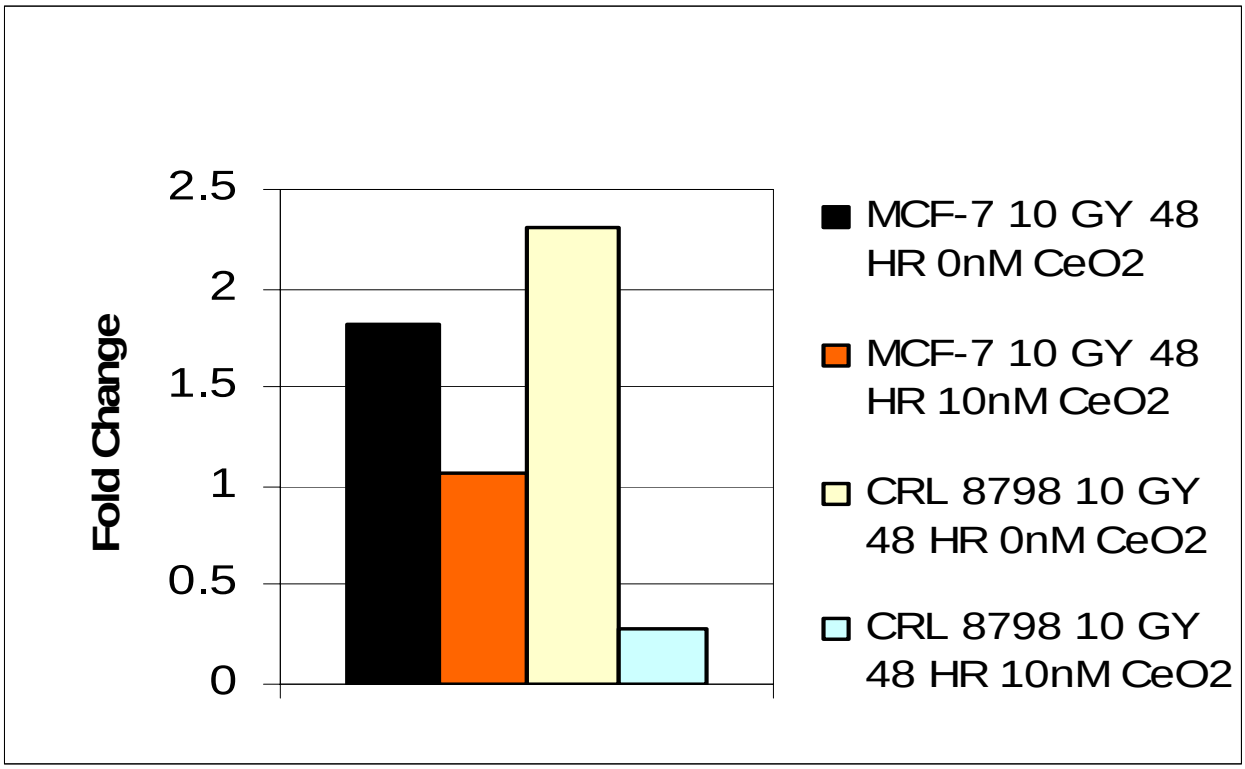

Figure 16. Protective effects of cerium oxide nanoparticles at 48 hours.

A comparison between normal breast cells (CRL 8798) and tumor breast cells (MCF-7) 48 hours after ionizing radiation. The percentage increase of TUNEL positive cells after a single dose of 10 Gy ionizing radiation was evaluated to compare the effects of the cerium oxide nanoparticles in normal and tumor breast cells. We observed a 2-fold increase in TUNEL positive cells on the untreated tumor cells and a 1-fold increase in the treated tumor breast cell lines. Also we observed a 2-fold increase in the untreated normal breast cell line and a 0.3 fold increase in the treated normal breast cell line.

Ce 142 uptake in MCF-7 and CRL 8798 cell lines measured by Induced Coupled Plasma-Mass Spectroscopy ICP-MS

To determine if the cerium oxide nanoparticles were present inside the cells we analyzed the samples for the presence of the element Ce (cerium). The presence of cerium oxide nanoparticles inside the cells, for both tumor cells and normal cells, was detected by induced coupled plasma-mass spectroscopy (ICP-MS). Cells were grown to confluency in 6 well plates. The nanoparticle solution was added to each well and the plates were incubated for 24 and 48 hours at 37 C and 5% CO2. After the incubation, the cells were removed by trypsinization and washed in Ca2+ and Mg2+ free PBS. The samples were kept at –80C. The cell numbers were normalized according to their protein concentration for each sample.

We detected the presence of ceria on all samples tested for the ceria isotope Ce142. At 24 hours after addition of the nanoceria, a concentration of 0.00049 ppm was detected in the normal cells and a concentration of 0.000427 ppm was detected in tumor cells. These differences in concentrations suggest that the nanoceria are more easily uptake by the normal cells than by the tumor cells. The same results are seen at the 48 hour time point. The concentrations at 48 hours are 0.000621 ppm in the normal cell line and 0.000592 ppm in the tumor cell line. These results show that the nanoparticle concentration inside the cell was higher in the normal cell line than in the tumor cell line at 48 hours. We also noted that the nanoparticle concentration in both cell lines increased in a time dependent manner. The normal cell line nanoparticle concentration increased from 0.00049 to 0.000621 ppm and in the tumor cells from 0.000427 to 0.000592 ppm. These observations suggest that the nanoparticle uptake by the cells is a time dependent process

and that possibly a prolonged incubation time can increase the protective effects of the nanoceria. The mode of the nanoceria entry into the cells remains unknown. There is electron microscopy evidence showing cerium oxide nanoparticles entering rat brain cells by phagocytosis, but more studies need to be conducted in order to elucidate this process.

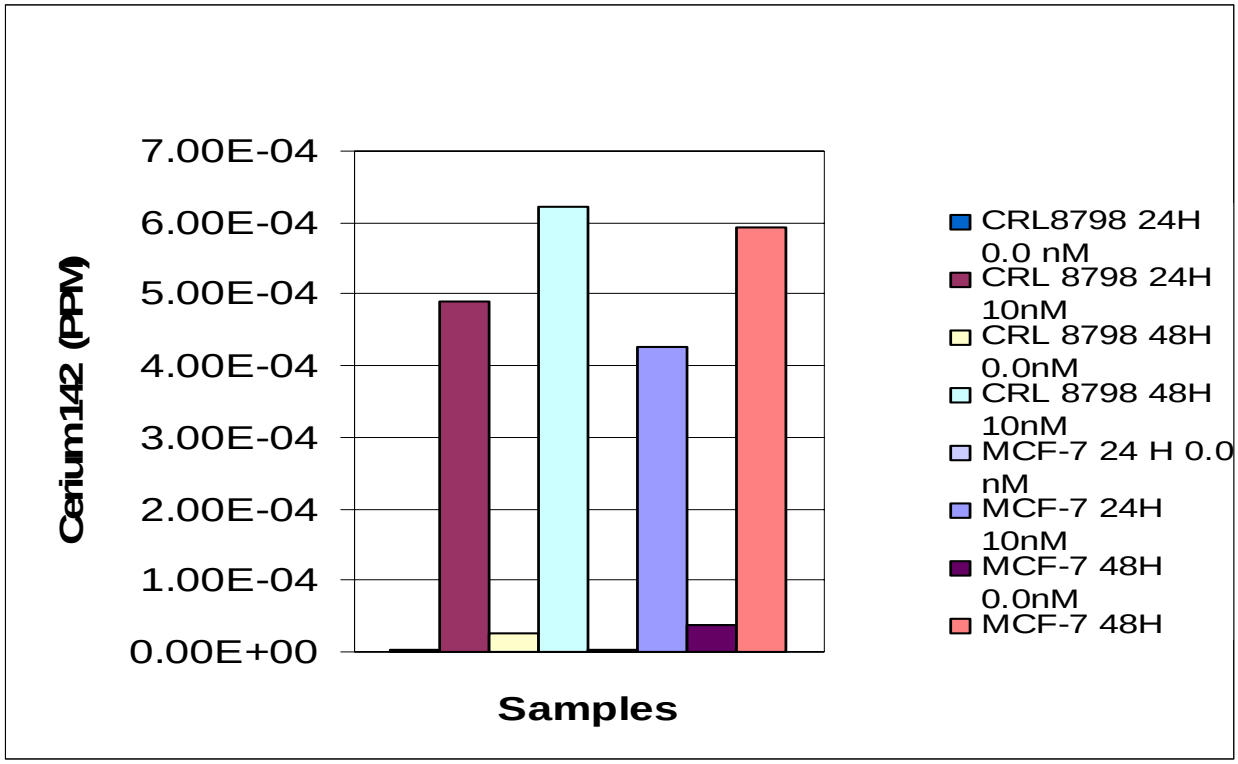

Figure 17. Ce 142 uptake in MCF-7 and CRL 8798 cell lines measured by ICP-MS.

Samples were analyzed by ICP/MS at the Notre Dame University, IN. The concentration of ceria was determined by analyzing the presence of the ceria isotope Ce 142 in the cell samples. All concentration were expressed in parts per million (PPM).

CONCLUSION

Effective long-lived radioprotectants with clinical applications are not well represented in most scientific literature. Amifostine is the only clinically relevant radioprotectant which was synthesized in the 1950's at the Walter Reed Army Research Facility. Cerium oxide nanoparticles show very promising characteristics that may allow them to be useful radioprotectant agents. Our studies have demonstrated a differential efficacy in normal versus tumor cells in culture. This efficacy could be due to differential intercellular activity or differences in nanoceria intake by the cells. Inter- and extracellular pH differences exist between tumor and normal tissues due to different metabolic activity of tumor cells[3]. These effects might play a role in the free-radical scavenging activities 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 free radical scavengers might protect the fewer damaged sites in the normal cells versus the more relaxed chromatin and greater number of targets of tumor cells and help account for this observed difference.

We propose that cerium oxide nanoparticles act as antioxidants due to the presence of the mixed valence states of Ce^{3+} and Ce^{4+} on the surface, (induced by the oxygen vacancies). We believe that 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 exhibit very low or no observed 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 play a role as an effective radioprotectant for normal tissues as well as offer differential protection to normal cells as compared to tumor cells after radiation. Further studies will determine the mechanism of this differential effect and determine the efficacy in animal models for ionizing radiation, both for general radioprotection as well as for radiation oncology applications.

LIST OF REFERENCES

1. Pradhan, D. S.; Nair, C. K. K.; Sreenivasan, A. *Proc. Ind. Natl. Sci. Acad*. **1973**, 39B, 516.

2. Grdina, D. J.; Thomas, J. S.; Kataoka, Y. *Oncology.* **2002**, 63 (Suppl. 2): 2.

3. Cohen-Jonathan, E.; Berhhard, E. J.; McKenna, W. G. Current *Opinion in Chemical Biology.* **1999**, 3, 77.

4. Eguchi, K.; Setoguchi, T.; Inoue, T.; Arai, H. *Solid State Ionics*. **1992**, 52, 165.

5. Tsunekawa, S. et al. *Materials Science Forum*. **1999**, 315, 439.

6. Izu, N.; Shin, W.; Matsubara, I.; Murayama, *N. Journal of Electroceramics*. **2004**, 13, 703.

7. Jasinski, P.; Suzuki, T.; Anderson, H. U. *Sensors and Actuators B-Chemical.* **2003**, 95, 73.

8. Masui, T.; Ozaki, T.; Machida, K.; Adachi, G. *Journal of Alloys and Compounds*. **2000**. 303, 49.

9. Spanier, J. E.; Robinson, R. D.; Zhang, F.; Chan, S. W.; Herman, I. P. *Physical Review.* **2001**, B 64, 245407-1.

10. Feng, Z. et al. *Applied Physics Letters.* **2002**, 80, 127.

11. Clark A.; Ellison, A.; Fry, R.; Merchant, S.; Kuiry, S.; Patil, S.; Seal, S.; Rzigalinski, B. Program No. 878.2. 2003 Abstract Viewer/Itinerary Planner. Washington, DC: *Society for Neuroscience*. **2003**, Online.

12. Patil, S.; Kuiry, S. C.; Seal, S.; Vanfleet, R. *Journal of Nanoparticle Research.* **2002**, 4, 433.

13. Wang, C.; Zhang, W.; Qian, Y. *Materials Science and Engineering*. **2002**. B94, 170.

14. Tsunekawa, S.; Sivamohan, R.; Ito, S.; Kasuya, A.; Fukuda, T. *Nanostructured Materials.* **1999**, 11, 141.

15. Deshpande, S.; Patil, S.; Kuchibhatla, S.; Seal S. *Applied Physics Letters.* **2005**, 87, 133113:1.

16. Constantini, M. et al. *Evaluation of Human Health Risk from Cerium Added to Deisel Fuel*, Health Effects Institute, Boston MA, Communication 9, **2001**.

17. *Development of Reference Doses and Reference Concentrations for Lanthanides*, Toxicology Excellence for Risk Assessment, The Bureau of Land Management, National Applies Resource Sciences Center, Amended Stage 2, November **1999**.

18. Nair, C. K. K. ; Parida, D. P. ; Nomura, T. J. *Radiat. Res.* **2001**, 42, 21.