ABSTRACT

COMPARISON OF TWO HUMAN CELL LINES FOLLOWING EXPOSURE TO LOW DOSE RADIATION AND TREATMENT WITH SOYBEAN MISO AND ITS ACTIVE INGREDIENT GENISTEIN

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Exposure to radiation is increasingly becoming a worldwide health issue. Because of this, the need for effective radioprotectors is critical. This need is not met by amifostine, the current "gold standard" in radioprotectors, due to its limited availability and potentially severe side effects. The fermented soy product miso has been credited with a host of beneficial effects, among which are radioprotection, and, as a common food, miso is readily available, known to be safe and is easily administered. As such, it is the hypothesis of this dissertation that miso may be an effective radioprotector. Unfortunately, few rigorous studies of miso's reputed actions have been undertaken. It is the objective of this investigation, therefore, to better identify and characterize the radioprotective and antioncogenic properties of miso and its most abundant active ingredient genistein. To accomplish this, experiments were designed using tumorigenic (PC3 cells) and non-tumorigenic (RWPE-1 cells) experimental prostate models to characterize the effects of miso and genistein both alone and following a series of low dose radiation exposures. Results demonstrated that neither miso nor genistein appeared to display radioprotective abilities in the radiation dose range used in this investigation. However, both compounds induced a strong growth inhibitory effect that appeared to synergize with radiation to significantly reduce cell survival in a manner that preferentially enhanced tumor cell kill over non-tumor cell kill, suggesting potential antioncogenic potential for these compounds.

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DEDICATION

This work is dedicated to the memory of my Grandma, Shirley Neurohr.

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LIST OF ABBREVIATIONS

7AAD	7-Aminoactinomycin D
AED	5-androstenediol
AFRRI	Armed Forces Radiobiology Research Institute
ANOVA	Analysis of variance
ATCC	American Type Tissue Culture
ATP	Adenosine triphosphate
cdc2	Cell dependent cyclins
CDK	Cyclin dependent kinase
CSC	Cancer stem cells
CT	Computed tomography
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRF	Dose reduction factor
G-CSF	Granulocyte colony-stimulating factor
GF-CFC	Granulocyte-macrophage colony forming cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPV	Human papilloma virus
i.p.	Intraperitoneal
IGRT	Image-guided radiation therapy
IL-1	Interleukin-1

IL-6	Interleukin-6
IMDM	Iscove's modified Dulbecco's medium
IMRT	Intensity-modulated radiation therapy
IU	International units
KSFM	Keratinocyte serum free media
LLC	Lewis lung carcinoma cells
M-CSF	Macrophage colony-stimulating factor
NC	Negative control
NCI	National Cancer Institute
OXM	Oxymetholone
PARP	Poly (ADP-ribose) polymerase
PBL	Human peripheral lymphocytes
PC	Positive control
PMVEC	Pulmonary microvascular endothelial cells
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RDD	Radiological Dispersion Device
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
s.c.	Subcutaneous
SEM	Standard error of mean
SF	Survival fraction
TBI	Total body irradiation

TMG	Tocopherol monoglucoside
TNF	Tumor necrosis factor
WBC	White blood cell

CHAPTER 1: INTRODUCTION

1.1 RADIATION INTERACTIONS IN BIOLOGICAL SYSTEMS

Radiation describes the transfer of energy to atomic species from either elementary particles or electromagnetic waves as they propagate through space [1]. It can consist of both non-ionizing and ionizing transfers as described below, but the term is often used informally as a synonym for the ionizing forms of radiation.

Non-Ionizing Radiation - Non-ionizing radiation refers to any type of electromagnetic radiation that does not carry enough energy to ionize atoms or molecules (i.e. to remove an electron from its atomic orbital) [2]. Examples of non-ionizing radiation include ultraviolet radiation, visible light, infrared radiation, microwaves, and radio waves. The energy of non-ionizing radiation is low, and much of their biological action is due to thermal effects created from the changes in rotational, vibrational or electronic valence configurations of molecules with which they interact [3]. Higher energy non-ionizing radiations such as visible and ultraviolet radiation may act through the production of photochemical reactions as well [2].

Ionizing Radiation - Ionization is a chemical process in which an electron(s) is removed from an atom. For radiation, this occurs following the collision of the atom with a photon or particle of sufficient energy to overcome the binding energy holding the electron within its atomic orbital [4]. Ionizing radiation can cause significant harm to biological systems through its production of unregulated chemical changes of important macromolecules within the cell. In particular, alterations in the macromolecule deoxyribonucleic acid (DNA) is important, since it is generally held to be the critical target of radiation-induced cellular damage [2]. If not repaired, unregulated changes in the DNA structure can lead to mutations, cancer and even cell death.

There are several forms of radiation that naturally exist which have the energy to ionize atoms and molecules. Within the electromagnetic spectrum, these include the high frequency radiations known as gamma rays and x-rays (wavelengths of 10^{-6} cm or smaller). Additionally, several types of high energy particles, such as electrons (negatively charged atomic particles), protons (positively charged atomic particles), alpha particles (nuclei of helium atoms), neutrons (uncharged atomic particles), and heavy charged ions (nuclei of elements such as carbon, neon and argon), can be ionizing [4].

The extent of the radiation-induced damage accrued within a cell is known to be a function of how much ionizing radiation is absorbed (absorbed dose is described in System International units of Gray or Gy) [5]. However, the type of radiation to which the atom is exposed also plays a considerable role. Specifically, different types of radiation exhibit different biological responses depending upon the density of their ionization events. This density of ionization is expressed in terms of Linear Energy Transfer (LET) and describes the energy transferred per unit length of ionization track [4]. The unit used for LET is the kiloelectron volt per micrometer (keV/μm) of material being traversed by the radiation [4]. In general, high LET radiations, such as protons, neutrons and alpha particles, generate significantly larger numbers of DNA lesions than do low LET radiations, such as gamma rays and x-rays, and, as a consequence, dose per dose, they result in significantly more biological damage [2]. To account for the differing biological responses from the various radiation types and to achieve an equivalent radiation dose (described in System International units of Sievert or Sv), a weighting coefficient known as the Quality Factor (QF) is multiplied by the absorbed dose [2].

Direct versus Indirect Action of Ionizing Radiation - As stated above, it is generally believed that the critical target leading to radiation-induced damage is the macromolecule

DNA[2]. This DNA damage comes about through two different processes – direct action and indirect action. Direct action occurs when radiation interacts directly with the DNA to cause changes in its structure (i.e. point mutations, DNA strand breaks, DNA crosslinks, and chromosome aberrations) [6]. These changes, if not repaired, can initiate a chain of events leading to cellular damage or death. Direct action predominates as the mode of cell killing following high LET radiation (i.e. protons, neutrons, alpha particles). Due to the difficulty in repairing DNA lesions arising from these densely ionizing radiations, this process is difficult to modify by chemical or physical means [2].

Alternatively, indirect action occurs from the production of highly reactive, chemically unstable free radicals within the cell that subsequently interact with the DNA to cause damage [7]. Briefly, ionization of an atom/molecule results in the creation of a free, negatively-charged electron and an ionized, positively-charged atom/molecule. Both of these entities are charged and possess unpaired electrons, making them, by definition, ion radicals, an intermediate in the formation of free radicals (uncharged atoms/molecules possessing an unpaired electron) [2]. In non-biological systems, the formation of free radicals is often of little concern. Once the radiation ceases, the electrons rejoin the atoms and no damage is done [7]. However, because of the critical role that free radicals play in metabolic regulation and cell signaling, their formation in biological systems can be disastrous, often leading to severe consequences [6].

Since roughly eighty percent of the cell is composed of water, much of the radiation damage done by indirect action occurs when radiation ionizes the water molecules of the cell – a process known as radiolysis [2]. Radiolysis of water leads to the production of the several free radicals, but of particular importance is the hydroxyl radical. The hydroxyl radical is highly reactive and can readily interact with DNA to cause structural change [7]. Moreover, it is

sufficiently long-lived to be able to diffuse a distance that is twice the diameter of the DNA molecule itself, thereby effectively increasing the "vulnerability zone" of the DNA [6]. Indeed, for low LET radiations such as x-rays and gamma rays, it is estimated that about two thirds of the radiation-induced damage in a cell is due to interaction with the hydroxyl radical, making indirect action the predominant mode of cell killing for these types of radiation [2]. Finally, because indirect action is due to free radical interactions, this mode of cell damage has the potential to be modified by chemical means [7]. Radioprotection can be enhanced by the use of free radical scavengers and radiosensitization can be improved by agents that prevent the neutralization of free radicals [6].

1.2. THE PROBLEM: INCREASING EXPOSURE TO LOW DOSES OF RADIATION

The use of ionizing radiation in medical procedures began shortly after the discovery of x-rays in 1895 by Wilhelm Roentgen. One year later, Leopold Freund utilized x-rays to successfully treat a hairy nevus [8]. In 1898, once Pierre and Marie Curie isolated the radioactive element radium, treatment with ionizing radiation was no longer limited to x-rays, but included radioisotopes as well [9]. During the twentieth century, advancements in technology and better understanding of radiation's cellular effects led to greater and greater use of diagnostic and therapeutic radiation. For example, the use of computed tomography (CT) scans in diagnostic radiology increased rapidly from 3 million scans in 1980 to approximately 62 million scans in 2006 [10]. Concern over the increasing number of CT scans is due to the increased radiation dose when compared with conventional x-ray doses. An adult abdominal CT delivers a dose that is approximately fifty times larger than the conventional anterior-posterior abdominal x-ray dose of 0.25 mGy [11]. Advancements in the treatment of cancer also increase

human exposures to low dose radiation (less than 1 Gray). Intensity-modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT) are better able to deliver targeted doses of radiation in the shape of the tumor, better sparing surrounding normal tissue from high doses when compared to conventional radiotherapy. However, the use of many fields in IMRT and IGRT causes a larger volume of normal tissue to be exposed to low dose radiation [12].

Nevertheless, historically, radiation exposures were predominantly limited to persons within the "first world" nations. However, in the twenty-first century, it is projected that underdeveloped countries will gain ever-increasing access to diagnostic and therapeutic radiation procedures, making exposure to radiation, especially low doses of radiation, a critical world health concern. Furthermore, increased radiation exposure to an ever expanding population throughout the world is not only the result of medical procedures. Nuclear power as an energy source is currently experiencing renewed interest resulting from concerns about climate change, instability in fossil fuel supplies, and the political motivation for energy security [13]. The use of nuclear power is predicted to increase from 2.7 trillion kilowatt-hours in 2006 to 3.8 trillion kilowatt-hours in 2030 [14]. Increased nuclear power usage carries with it the threat of another nuclear accident like Chernobyl or Fukushima.

The events at Chernobyl began on April 25th, 1986 with the operators disabling safety systems to run a test program that violated the prescribed operating limits, causing the reactor to go into an unstable state. The "reactor shut-down" button was pushed on April 26th, but a very strong power spike was initiated by runaway fission reactions causing explosive destruction of fuel channels. The core cavity lid (weighing 1000 tons) was lifted and overturned by escaping steam and gases. Another explosion occurred, and the reactor was completely destroyed. Evaporated fuel and fuel fragments were spewed into the air and further radioactive releases

occurred due to the fire (lasting for 10 days) that started in the remaining graphite [15]. In total, 800,000 TBq of Iodine-131, 85,000 TBq of Cesium-137, and 100% of the noble gasses (such as Xenon-133) were released into the atmosphere [16].

In contrast to Chernobyl, where human error played a major role when the event started, Fukushima began with a combination of two natural events an earthquake and a tsunami. While the reactors could have withstood the earthquake, the resulting tsunami (about 14 meters high) overwhelmed the reactors that were designed to withstand wave heights of only 5.7 meters [15]. The emergency diesel engines, power distribution, instrumentation, and control equipment was inundated with salt water. The loss of the power and equipment caused the cooling in reactors 1 through 3 to be lost. Large amounts of hydrogen and fission products were released into the containments, and these containments developed leaks. Leakage of hydrogen gas caused explosions which destroyed the upper parts of the reactor buildings in units 1, 3, and 4. The previously outlined events led to the release of 150,000 TBq of I-131 and 12,000 TBq of Cs-137 [17] and the first few months following the event an additional 4,000 TBq of Cs-137 were released into the sea.

The scale of possible radiological accidents can also be understood by examining an incident that occurred in Goiania, Brazil in 1987. Two people entered an abandoned clinic to remove a stainless steel cylinder from a cancer therapy machine. The cylinder was then sold to a junkyard, where it was dismantled to reveal a blue salt-like substance. This substance was later identified as approximately 1400 Curies (5.18×10^{13} Bq) of cesium-137 [18]. The cesium was broken into little pieces with various people taking it home, and pieces were even given to the workers' children as playthings. As a consequence of this accident, 249 people were contaminated either internally or externally. Four people died within four weeks after hospital

admission and one person lost an arm to amputation [19]. This case demonstrates how inadvertently a radiological incident can occur and how serious and widespread the effects can be, especially to unwitting populations.

Accumulating nuclear waste is also another potential source for exposure to low dose radiation, with no country in the world yet building a permanent geologic repository [13]. If a permanent solution is found for the disposal of the waste, the disposition of the waste is unlikely to be in the same area as the power plant, creating further exposure concerns from waste transportation and corresponding population exposure, as the nuclear waste is moved from one area to another, once again, increasing accident potential.

Finally, culminating with the events of September 11, 2001, the general public has become aware of terrorism as a harsh reality, not only in the United States, but worldwide. Radiological terrorism must be considered in a current listing of concerns when examining potential sources of low dose radiation and human exposure. Radiological Dispersion Devices (RDDs), also known as dirty bombs, are believed to be the most probable radiation weapons for terrorists, due to their relatively simple technology and the widespread use of RDD-adaptable radioactive materials derived from such industries as oil drilling, medicine and scientific research [3, 20]. The RDD concept is that a terrorist could use conventional explosives to disperse radioactive materials with the potential for exposing large populations of people to varying doses of ionizing radiation. A serious threat of radiological terrorism occurred in 2002, when an individual was detained on suspicion of intending to deploy a radiological dispersion device (RDD) in the United States [21].

1.3. THE CHALLENGE: FINDING EFFECTIVE, READILY AVAILABLE RADIOPROTECTORS

Due to the concerns about increased radiation exposure, it is necessary for effective radioprotective compounds to be found that can mitigate, or at least attenuate, the deleterious effects of ionizing radiation. In the opinion of one prominent researcher, S. Hosseinimeher, an ideal radioprotective agent would provide significant protection to a majority of organs, be easily administered (i.e. orally), have a low toxicity, have a protective time-window, be stable in both the bulk active product and the formulated compound, and also be compatible with a wide range of drugs [22]. Several compounds currently under investigation include thiol and synthetic radioprotectors, nitroxides, bisbenzimidazol, cytokines, immunomodulators, natural antioxidants, and herbal medicines.

1.3.1 Thiol and Synthetic Radioprotectors

The creation of reactive free radicals, such as hydroxyl radicals, peroxyl radicals, alkoxyl radicals, and nitric oxide, occurs when ionizing radiation interacts with the water in a cell [23]. Under these conditions, free radicals cause damage to critical macromolecules such as DNA. Damage to the DNA is a leading cause of cell killing, carcinogenesis, and mutations. To protect against this type of damage, one mechanism of radioprotection is the use of free radical scavengers.

Thiols are molecules that contain free, or potentially free, sulfhydryl groups in their structure that are free radical scavengers for both ionizing radiation and chemotherapy agents. Thiols are able to donate a hydrogen atom to reduce the free radical [2].

In 1948, one of the first accounts of using thiols as radioprotectors was recorded. H.M. Patt discovered that cysteine, a thiol, protected mice from the effects of whole body exposure to

x-rays if injected or ingested in large amounts before the radiation exposure [2, 24]. There were promising results; however, when appropriate doses were administered for radioprotection to occur, cysteine was found to induce nausea and other severe side effects. Cysteamine, a degradation product of cysteine, also demonstrated the ability to protect animals from whole body radiation exposure [2], but cysteamine also became toxic at doses necessary to protect from ionizing radiation.

In 1959, a research program developed by the U.S. Army at the Walter Reed Institute of Research sought to identify and synthesize new agents capable of protecting against ionizing radiation with less toxicity. Approximately 4400 compounds were developed and tested by 1973 [22]. One of the most effective of these compounds is WR-2721, also known as amifostine or by the company MedImmune under the brand name Ethyol. Amifostine is a prodrug, i.e. a drug that must undergo chemical conversion by metabolic processes before becoming active. In the case of amifostine, the thioester bond is cleaved by a cell membrane alkaline phosphatase yielding a free thiol, the active metabolite WR-1065 [25]. Enhancing its free radical scavenging abilities, amifostine is able to cytoprotect through at least one other mechanism which is its binding to the active species of alkylating agents leading to detoxification [26]. After exposure to ^{60}Co Υ-radiation doses ranging from 1.5 Gy to 6.0 Gy, amifostine reduced radiation-induced apoptosis in thymic small lymphocytes [27]. These mechanisms make amifostine an effective radioprotective candidate, and the use of it began soon after its discovery, with amifostine being the compound carried by astronauts on trips to the moon in case of a solar radiation event [2].

Currently, amifostine is the only cytoprotective agent that is approved by the Food and Drug Administration specifically for use as a radioprotector [28]. For example, the DRF (Dose Reduction Factor) for murine hematopoietic tissue is as high as 2.7 [2]. The DRF is determined

by dividing the dose of radiation in the presence of the drug by the dose of radiation in the absence of the drug to produce a given lethality. For example, mice injected with a dose of 900 mg/kg of amifostine require a dose of x-rays 2.7 times that of control animals to produce the same mortality rate [2]. Amifostine, used in clinical settings, is able to reduce the incidence and severity of acute and chronic xerostomia in patients with head or neck cancer without affecting the efficacy of the radiation [29]. For maximum radioprotection, amifostine is administered intravenously (IV) twenty to thirty minutes before each radiotherapy fraction [30]. Trained medical personnel must also watch the patient for any side effects from amifostine including acute hypotension, severe nausea, vomiting and allergic reactions [29]. In the case of a widespread nuclear accident or terrorist act, problematic areas would be accessing prescription drugs and immediately locating trained medical personnel to administer the drug.

Even if amifostine could be easily administered and the toxicity was reduced, amifostine has other significant disadvantages, such as the inability to protect all human organ systems. For example, the central nervous system is wholly unprotected because amifostine is unable to pass through the blood-brain barrier [31]. The hematopoietic system has a DRF of 2.7 in comparison to the gastrointestinal system DRF of 1.8 in mice [2]. The ideal radioprotector would provide radioprotection to all organ systems for maximum benefit. Another complication with amifostine is the limited time window of administration. Maximum radioprotection occurs when amifostine is administered before the radiation exposure. Therefore, as advance notice of a nuclear accident or a radiological terrorist attack is unlikely or impossible, amifostine is not best suited. Another drawback limiting the use of amifostine is the high cost of the drug. At an estimated $400 per dose in 2005 [32], this drug is too expensive for almost everybody in the general population, severely reducing mass access. While amifostine has potential, a better

candidate would be less toxic, easier to administer, protective all organ systems, and be equally effective before and after radiation exposure.

1.3.2 Nitroxides

Nitroxides are stable free radical compounds used as a contrast agent for Magnetic Resonance Imaging (MRI) and as biophysical tools for electronic spin resonance spectroscopic studies and spin labeling oximetry [33]. In addition to their use as biophysical tools, nitroxides were observed to interact with other free radicals, including those that may be created from ionizing radiation. In this classification of radioprotectors, the stable free radical Tempol (4-hydroxy-2,3,6,6-tetramethyl piperidine-1-oxyl) is the compound generating the most interest. The antioxidant properties of Tempol result from several different mechanisms including superoxide dismutase-like activity [34] and the scavenging of other free radicals. Tempol provides protection *in vitro* for mammalian cells against radiation-induced cytotoxicity under aerobic conditions [35], and is also able to provide protection *in vivo* against whole body irradiation [36]. Tempol, however, has significant side effects including hypotension, increased heart rate, and seizure activity at doses necessary for radioprotection [22]. Currently, topical or regional administration methods are being explored as a way to lessen the toxicity of Tempol [37]. Should alternate methods of administration become feasible, Tempol's short time window of effect remains a key factor in consideration for its use as a radioprotector.

New studies focusing on the reduced form of Tempol, Tempol-H, are directing the laboratory investigation toward the drug being tolerated better, while still providing significant radioprotection. Radioprotection has been observed in studies where sixty percent of mice treated with Tempol-H survived an 11 Gy dose while ten percent survived 30 days following a 13 Gy dose [33]. In contrast, control mice survived after exposure to 9 Gy of total body

irradiation (TBI) while none survived a 10 Gy TBI dose. This study demonstrates significant radioprotection as well as the ability *in vivo* to convert the reduced form of the compound to the active, oxidized compound. Even though radioprotection was observed with Tempol-H, the toxicity profile was similar to that of Tempol. While Tempol and Tempol-H both provide significant radioprotection, widespread use as radioprotectors require less toxicity and easier routes of administration (i.e. orally).

1.3.3 Bisbenzimidazol

Bisbenzimidazol compounds are used as reagents for the *in vitro* estimation of DNA concentration and for histological applications because of their ability to strongly fluoresce upon binding to DNA [22]. In addition to the use of these compounds as markers, in 1984, one particular bisbenzimidazol compound, Hoechst 33342, was reported to radioprotect human colon adenocarcinoma cells [38]. Hoechst 33342 was also radioprotective *in vivo* with intravenous administration thirty minutes prior to a single radiation dose of 12 Gy by ^{137}Cs-ϒ producing a DRF of 1.2 in a mouse lung model [39]. Despite the radioprotection observed, the compound also proved to be mutagenic and cytotoxic above concentrations of 20 – 30 μM [39, 40].

In an effort to reduce the toxicity to normal cells, derivatives of Hoechst 33342 have been explored. One of these derivatives, methylproamine, was shown to be 100 times more effective than WR-1065 (the active metabolite of amifostine) in Chinese Hamster lung fibroblasts (the V79 cell line) [41]; however, further research is necessary to explore toxicity and efficacy in animals.

1.3.4 Cytokines

Depending on the dose received, exposure to ionizing radiation may lead to death through three different syndromes. The first syndrome is termed the cerebrovascular syndrome because 24-48 hours after radiation exposure, death occurs due to neurologic and cardiovascular breakdown after a TBI γ-radiation dose of 100 Gy or more [2]. The gastrointestinal syndrome occurs at doses greater than 10 Gy, and death occurs from three to ten days after exposure due to extensive bloody diarrhea and destruction of the gastrointestinal mucosa. Doses of approximately 2 to 10 Gy can lead to death due to the hematopoietic syndrome, although doses of 2 to 5 Gy are survivable, especially if good medical care is provided [2]. Specifically, in this syndrome, ionizing radiation affects the hematopoietic system by reducing the numbers of neutrophils and platelets. The decrease in the number of neutrophils and platelets may lead to septicemia, hemorrhage, anemia, and death [22]. In order to mitigate the effect of ionizing radiation on the hematopoietic system, investigations into novel radioprotectors have focused on the stimulation, maintenance, and proliferation of progenitor cells from the bone marrow. Cytokines, a group of soluble glycoproteins and low molecular weight peptides, stimulate cell proliferation and differentiation in hematopoietic and lymphoid tissues [42] which has led to investigations into the radioprotective qualities of cytokines.

In animal models, the most extensively studied cytokine for radioprotection is Interleukin-1 (IL-1). IL-1 is mainly produced by monocytes and macrophages in response to endotoxins, other cytokines, and microbial or viral agents [42]. IL-1 plays an important role in regulating hemopoiesis by directly stimulating the most primitive stem cells. Indirectly, it increases production of other hematopoietic factors such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage

colony-stimulating factor (M-CSF), and interleukin-6 (IL-6) [43]. Treatment with IL-1 alone is showing promising results. Maximal radioprotection was observed when mice were administered 0.1 μg IL-1 twenty-four hours prior to a gamma radiation exposure of 9.5 Gy. In this case, 80% of the treated group survived compared to 0% of the control group [44].

1.3.5 Immunomodulators

The cytotoxicity of cytokines has led researchers to investigate the use of non-cytokine drugs that stimulate hematopoietic stem cells. The drugs, known as immunomodulators, may increase cytokine activity that can stimulate growth, differentiation, and proliferation of hematopoietic progenitor and stem cells. By protecting the hematopoietic system, the immunomodulators appear to protect and repair through enhanced production of bone marrow cells, circulating granulocytes, lymphocytes, and platelets [43] .

A group of water-soluble polysaccharides, β-glucans, act as biological response modifiers by regulating the immune system [22]. The main mechanism of glucan action appears to be polysaccharide-induced stimulation of macrophage and granulocyte production [45]. This was further proved when glucan administration increased the number of endogenous pluripotent hematopoietic stem cells that had been depleted by 6.5 Gy of ^{60}Co irradiation in mice [46]. In this case, the most potent radioprotection was observed when glucan was administered one day before irradiation [46]. In contrast, glucan administered 10 minutes before gamma irradiation resulted in a DRF of 1.4, which is lower than WR-2721 with a DRF of 2.2 [22]. Studies then led to the idea of combining different radioprotective agents, in this case glucan and WR-2721, to maximize protection and possibly reduce the toxicity of WR-2721. Mice exposed to 6.87 Gy of X-irradiation, treated with both glucan and WR-2721, had a DRF of 1.7 [45]. The DRF

increased with the combination of glucan and WR-2721 (DRF 1.7), however the isolated WR-2721 alone generates a greater DRF of 2.2.

Another immunomodulator showing promise as a radioprotector is the anabolic-androgenic steroid oxymetholone (OXM) [22]. Mice, receiving an oral dose of OXM 24 hours prior to gamma irradiation with 8 Gy, had a survival rate of 75% when compared to the control group survival at 15%, a DRF of 1.14 [47]. Along with increasing the survival rate of irradiated mice, oral administration of OXM increased platelet and red blood cell (RBC) number, although white blood cell cells (WBC) were not affected. The advantages over amifostine are that OXM is less toxic [48], effective when administered orally, and is effective over a longer time period [47]. The drawbacks are that the exact mechanism behind the action of OXM is not known, it is not readily available to the general public, and has a lower DRF than amifostine.

The Walter Reed Army Institute of Research and the Armed Forces Radiobiology Research Institute (AFRRI) are credited with investigating over 4000 compounds to use as radioprotectors over the past 40 years [49]. AFRRI initiated the evaluation of androstene steroids based on their ability to normalize cytokine expression patterns and immune function after severe burn injury [50]. A natural hormone produced in the reticularis of the adrenal cortex, 5-androstenediol (AED) demonstrated a strong ability to increase the number of circulating neutrophils, platelets, and natural killer cells; stimulate myelpoiesis; and enhance the resistance to infection in mice following total body irradiation [51, 52].

AED is not limited in its ability to stimulate the hematopoietic system, but it also demonstrates the ability to radioprotect. Mice administered 160 mg/kg of AED by subcutaneous injection 24 hours before gamma irradiation significantly improved the survival rate with a DRF of 1.26 [51]. The next step evaluated the oral efficacy of AED as the preferred method of

administration for the general public. When mice were orally administered 1600 mg/kg of AED 24 hours prior to gamma irradiation (11.0 Gy), the survival rate increased to 60% versus 10% of the control group [53]. Another benefit to using AED is low toxicity, as determined from histopathology and clinical chemistry data [53].

Although several immunomodulators yield promising results in the area of radioprotection, research towards the ideal radioprotector continues today. An ideal radioprotector protects all organ systems, not just the hematopoietic system. Oral or topical administration serves as the quickest and easiest method for administering drugs for the lay public in case of an emergency; although none of these immunomodulators are readily available to the general public. At present, it is mandatory to obtain either a prescription or to be a researcher who has access to these compounds. Ideally, radioprotectors would have low to moderate toxicity, so the treatment would not outweigh the benefit. In the case of a widespread nuclear accident, radiological event, or terrorist event, access to the compounds, distribution, and administration would be difficult, at best.

1.3.6 Natural Antioxidants and Herbal Medicines

Several vitamins scavenge Reactive Oxygen Species (ROS) and upregulate the activities of antioxidant enzymes, with vitamin E (α-tocopherol) recognized as one of key importance [54]. Vitamin E is an antioxidant present *in vivo* that protects critical membrane components, such as phospholipids, transmembrane lipoproteins, and glycoproteins, from the free radicals produced by a variety of different exogenous and endogenous sources [55]. Due to its free radical scavenging abilities, its natural occurrence, and lack of toxicity at high doses [56], vitamin E was investigated as a viable radioprotector. Vitamin E was administered at a rate of 400 International Units (IU)/ kg to $CD2F_1$ mice by subcutaneous injection 24 hours prior to ^{60}Co irradiation at a

dose of 10.5 Gy, and the survival rate increased from 4% in the vehicle control treated group to 79% in the treated group [55]. Thirty day survival was also increased when vitamin E was delivered by s.c. injection 24 hours prior to irradiation with ^{60}Co, yielding a DRF of 1.23 [57]. Vitamin E injected s.c., either 1 hour before or within 15 minutes of irradiation with ^{60}Co, significantly increased 30-day survival in CD2F1 mice, with a DRF of 1.11 observed [58]. Vitamin E administered orally, however, did not affect survival when exposed to the same irradiation conditions as previously mentioned [55]. A derivative of vitamin E, a water-soluble derivative called tocopherol monoglucoside (TMG), was also investigated as a potential radioprotective agent. A DRF of 1.09 was obtained when mice were injected i.p. within 10 minutes of lethal irradiation [59]. These two compounds demonstrated the ability to radioprotect before and after -radiation exposure, although the DRF is lower than that of amifostine (DRF 2.7 in mice) [2]. Unlike amifostine, toxicity was not an issue with these two compounds.

Selenomethionine, a naturally occurring derivative of selenium, has a low toxicity and is found in soy, grains, legumes, and selenium-enriched yeast [60]. When administered i.p. at 24 hours prior, 1 hour prior, and 15 minutes after radiation exposure to ^{60}Co at a low dose rate, equal protection was observed. Specifically, no control animals survived after 10 Gy, whereas one-third of the treated animals survived regardless of whether the selenomethionine was administered 24 hours prior or 1 hour post radiation [61]. A similar degree of protection was achieved with sodium selenite, but it was more toxic than selenomethionine [58].

In addition to the radioprotective abilities of selenomethionine, it is also known to possess antioncogenic abilities. Concentrated research is necessary to determine the mechanism(s) behind the antioncogenic properties of selenium compounds [62]. Three possible mechanisms for their protective properties are: 1) cytosine methyltransferase inhibition [63]; 2)

control of tumor angiogenesis [64]; and 3) inhibition of carcinogen bioactivation [62]. The molecular genetic determinants of selenium action are also not known, and further studies are necessary to reveal the exact underlying mechanisms.

Melatonin, a hormone produced by the pineal gland, is commonly used as a self-administrated sleep-inducing medication, and some studies suggest it is capable of scavenging hydroxyl and peroxyl radicals and peroxynitrite anions [65]. Considering its low toxicity [66], scientists began to investigate the radioprotective abilities of melatonin. CD2-F1 mice exposed to 8.15 Gy of radiation resulted in survival of only 45-50%; however, when the mice were pretreated with melatonin at a dose of 125 mg/kg body weight, the survival was increased to 60%. When the increased melatonin dose of 250 mg/kg body weight was administered, the survival increased to 85% [67]. When a single oral dose of melatonin (300 mg) was administered to healthy human volunteers, the number of chromosomal aberrations and micronuclei induced by irradiation in cultured lymphocytes decreased significantly and time-dependently as compared with similarly irradiated lymphocytes in the blood collected at 0 hours [68]. While melatonin demonstrates significant advantages, the side effects of melatonin are sleep-induction and a mild hypotensive effect. This effect is intensified in hypotensive patients being treated with nifedipine [69]. It can be concluded, once again, that while melatonin's qualities meet some aspects of the ideal radioprotector, drug interaction has to be added as a direct concern in the quest to find a drug to attenuate and mitigate radiation-induced damage.

Curcumin, the active ingredient in the dietary spice turmeric, possesses anti-inflammatory, antioxidant, anticarcinogenic, hepatoprotective, and thrombosuppressive, properties [70-74]. In addition to these properties, curcumin has also been reported to be a radioprotective compound. Cultured peripheral blood lymphocytes were treated with doses of

curcumin ranging from 1μg/ml to 10μg/ml thirty minutes prior to exposure to γ-radiation. Pretreatment with curcumin at a dose of 10μg/ml protected the lymphocytes against radiation damage up to 4 Gy [75]. The radioprotective effect was attributed to decreases in lipid peroxidation and an increased antioxidant status [75]. The radioprotective ability of curcumin is also observed *in* vivo. Female C57BL/6 mice aged 6-10 weeks fed a 5% curcumin (in standard mouse chow) diet were exposed to a single x-ray dose of 13.5 Gy to the thorax. Mice fed the curcumin diet had a 45% survival rate which was a significantly higher rate compared the 23% survival rate observed in the radiation alone group [76]. An ideal radioprotector would spare normal tissue, but not tumor tissue. Lewis lung carcinoma cells (LLC) and pulmonary microvascular endothelial cells (PMVEC) were pretreated with curcumin at a dose of either 10μM or 25μM for 4 hours prior to irradiation with a Mark 1 Cesium irradiator at doses of 0, 2, 4, and 6 Gy. One hour after radiation exposure, the curcumin was removed by replacing the growth medium with fresh medium. Curcumin treated normal cells (PMVEC) showed no significant decrease in cell survival when compared to radiation alone [76]. Pretreatment with curcumin significantly decreased cell survival when combined with radiation on the LLC tumor cells (i.e. at 6 Gy treatment with radiation alone reduced the survival fraction to 0.1 while treatment with 10μM reduced the survival fraction to 0.02 and treatment with 25μM reduced the survival fraction to 0.005) [76]. Currently, clinical trials are under way to investigate the efficacy of curcumin in humans.

Ginseng, a term that refers to both the North American ginseng (*Panax quinquefolius*) and the Asian ginseng (*Panax ginseng*), is reported to have a host of beneficial effects including antiaging, antidiabetic, anticarcinogenic, analgesic, antistress, and antifatigue properties as well as promotion of DNA, RNA, and protein synthesis [77-79]. These beneficial properties have

been linked to the antioxidant abilities of ginseng [78-80]. Human peripheral lymphocytes (PBL) treated with North American ginseng extract (NAGE) were exposed to ^{137}Cs γ-radiation doses of 1 and 2 Gy. At both time points studied (0 and 24 hours prior to irradiation), a reduction in the number of micronuclei yield was observed when compared to radiation alone; at 1 Gy, the reduction was 51.1% and at 2 Gy the reduction was 49.1% [81].

Ginsan, a purified polysaccharide extract from the roots of panax ginseng, is another possible radioprotector that stimulates endogenous production of cytokines [22]. In studies examining the effect of ginsan on irradiated mice, it was found that ginsan administered 24 hours prior to lethal irradiation, shows an increased survival rate with a DRF of 1.45 [82]. Ginsan also stimulates a significant increase in the numbers of bone marrow cells, spleen cells, granulocyte-macrophage colony forming cells (GF-CFC), circulating neutrophils, lymphocytes, and platelets in irradiated mice [82]. The maximum efficacy for ginsan's effectiveness comes about when administered by intraperitoneal (i.p.) injection 24 hours before irradiation. Further studies are necessary to examine the systemic toxicity with oral delivery, the most desired method of administration for radioprotection.

1.4 SOY PRODUCTS AND COMPOUNDS

Since the first recorded record in 700 BC [83], miso continues to be a part of the daily diet of the Japanese population [84]. Miso is a Japanese food or seasoning produced by fermenting soybeans, most commonly, with koji mold (*Aspergillus oryzae*) and cultivated on steamed rice or barley [84]. Consumption of soy foods such as miso is correlated with reduced risk of cardiovascular disease [85] and a reduction in the potential for bone loss in healthy, postmenopausal women [86, 87]. The use of miso as a possible mitigator of radiation damage

was first documented in 1945. After the atomic bomb was dropped on the city of Nagasaki, a hospital located 1 mile from the epicenter was destroyed. The staff of this hospital, including Dr. Shinichiro Akizuki, worked closely with the fallout victims in areas of the city that were heavily damaged and highly radioactive. Dr. Akizuki and his staff did not suffer from expected effects of the radiation and Dr. Akizuki attributed this phenomenon to the fact that he and his staff regularly consumed miso soup [83]. Few rigorous studies have been conducted on the possible radioprotective abilities of whole miso, the living cultures with the koji mold, *in vivo* or *in vitro*.

Research has rather concentrated on the effect of the isolated soy isoflavones present in soy miso. Isoflavones are diphenolic compounds with a similar chemical structure to estrogen and are almost exclusively confined to being present in the legume family [88]. Of particular interest is genistein (4',5,7-trihydroxyflavone), a compound naturally occurring in soybeans. Scientific findings demonstrate a wide range of benefits ranging from improvements in cardiovascular disease, high cholesterol, and osteoporosis [89]. Additionally, genistein is under investigation as a possible radioprotector. When examining overall survival in mice, a single s.c. injection of genistein twenty-four hours prior to a gamma radiation dose of 9.5 Gy significantly increased 30-day survival with maximum radioprotection observed at a genistein dose of 200 mg/kg. A DRF of 1.16 was calculated, with 91% of treated mice showing an increased survival rate when compared to only 15% surviving in the control vehicle treated group [90]. No radioprotection was observed when genistein was administered 1 hour prior to gamma radiation exposure [90]. Male BALB/c mice were orally administered 160 mg/kg of genistein every day for 7 days prior to a gamma radiation dose of 6.0 Gy. The mice administered genistein had longer survival times with 30-day survival being increased when compared to control [91]. In another study, female C57BL/6J mice were injected s.c. with 200 mg/kg of genistein 24 hours

prior to irradiation with ^{60}Co at a dose of 7.75 Gy. Thirty days after irradiation, 92% of the genistein treated mice were alive compared to only 23% of the control mice [92].

1.5 OTHER RADIOMODULATING EFFECTS OF SOY PRODUCTS AND COMPOUNDS

Cancer is a growing health problem around the world, partly due to a steady increase in life expectancy, increasing urbanization and the subsequent changes in environmental and lifestyle conditions [6]. Over the past several decades, epidemiological evidence and laboratory research has documented convincing evidence that the isoflavones in soy contribute to lower rates of prostate and breast cancers in Asian cultures in comparison to Western populations [93]. For this reason, the antioncogenic properties of soy miso and its isoflavones is studied.

One particular research group led by J. Raffoul at Wayne State University in Detroit, Michigan, has developed a compound that simulates the composition of soy isoflavones present in soy foods. This compound is composed of 43% genistein, 21% daidzein, 2% glycitein, 2.5% protein, 11.9% fat, 1.7% water, and the remaining 17.9% is carbohydrates [94]. The artificial soy isoflavone compound, once dissolved in sodium carbonate (Na_2Co_3), can then be used for *in vitro* or *in vivo* experiments. These studies have demonstrated that the soy isoflavone compound is equally as effective as pure genistein in causing apoptotic cell death, inhibiting prostate cancer cell growth, and potentiating radiation-induced cell killing *in vitro* [95, 96]. In *in vivo* experiments using an orthotopic PC3 model, the combination of soy and radiation led to a greater control of primary tumor growth than in soy or radiation treatments alone [97].

Genistein, the most abundant active ingredient in soy miso, has also been studied. One of the most remarkable properties of treatment with genistein is growth inhibition in a variety of different cell lines. Mouse embryonic fibroblasts (NIH 3T3), incubated with genistein doses

ranging from 0 – 180 μM for 24 hours, demonstrated reduced cell viability. The reduced viability was observed above concentrations of 7.5 μM with cell viability reduced to 50% at a concentration of 41.5 μM and to 95% at 180 μM [98]. Growth inhibition is enhanced when genistein is combined with radiation. PC3 cells treated with 15 μM/L genistein and a single 300 cGy photon irradiation from a ^{60}Co unit resulted in a 69% growth inhibition when compared to 50% growth inhibition with only a single dose of 300 cGy [96]. This effect has also been demonstrated in human cervical cancer cells, ME180. Pre-treated for 48 hours with genistein, less than 5% of ME180 cells survived radiation doses of 200-800 cGy [99].

Growth inhibition shown after treatment with genistein is attributed to two different mechanisms: a cell cycle block in G_2/M and apoptosis. A dose-dependent decrease in cyclin B_1, cdc2 expression, and cdc2 kinase activity is observed with increasing genistein dose in the prostate cancer cell lines PC3 and LNCaP [100]; the human lung cancer cell line H460 [101]; a head and neck squamous cell carcinoma cell line, HN4 [102]; and normal breast epithelial cells, MCF-10F [103]. The decrease in cyclin B_1, cdc2 expression, and cdc2 kinase activity correspond to a cell cycle block in G_2/M. Investigations into other possible mechanisms behind the cell cycle block are still on-going.

Apoptosis is also believed to be another reason mechanism of growth inhibition. PC3 cells pretreated with 30 μM genistein for 24 hours followed by 3 Gy radiation dose from ^{60}Co were analyzed for the cleaved poly (ADP-ribose) polymerase (PARP) protein that is an indicator of apoptosis. Cleaved PARP expression was 5.6-fold higher in cells treated with genistein and radiation, compared to 3.6-fold fold higher expression in radiation alone, and 1.8-fold higher expression with genistein alone when compared to control cells [95]. The technique of flow cytometric analysis using 7AAD (7-Aminoactinomycin D) staining to detect and quantify

apoptosis has also been used [104]. Use of these different techniques has demonstrated that apoptosis occurs in breast cancer cells (MDA-MB-231, MDA-MB-435, and MCF-7); prostate cancer cells (PC3 and LNCaP); non-small-cell lung cancer cells (H460 and H322); and head and neck squamous carcinoma cells (HN4) treated with genistein for 48 hours [105]. Furthermore, studies demonstrated that if the previously mentioned cells were treated with genistein for 72 hours, the number of apoptotic cells increased even more [100-102, 105-109].

Genistein demonstrates a significant growth inhibitory effect on a variety of different cancer cells lines at doses greater than 10 μM. In contrast, genistein doses below 10 μM have shown a growth stimulatory effect. In one study, a human breast cancer carcinoma cell line, MCF-7, was treated with genistein in doses ranging from 0.01 - 100 μM. Maximum growth stimulation, approximately 3-fold higher than control, was observed at 1 μM and was sustained through doses of 10 μM [110]. In contrast, the higher doses of genistein caused growth inhibition in the same study. In a human ductal breast epithelial tumor cell line, T47D, growth was stimulated when treated with genistein doses ranging from 10 nM – 10 μM and growth inhibition was observed in genistein doses greater than 20 μM [111]. Clearly, genistein action in the cell is multifaceted and careful consideration of dosage is necessary.

1.6 CELLULAR RESPONSE TO RADIATION AND CHEMICAL STRESS

1.6.1 Cell Cycle Block

Cells have developed complex mechanisms to cope with damaging agents such as ionizing radiation and chemotherapeutic agents. One of these mechanisms is the ability of the cell to halt cell cycle progression to repair DNA damage before the cell enters into mitosis. As shown in Figure 1.2, the cell cycle consists of four distinct phases: G_1, G_2, S, and M. Quiescent cells, which are cells that are not progressing through the cell cycle, are designated to be in G_0.

Cells in G_1 are growing in size and synthesizing proteins and mRNA that are required for DNA synthesis. In S phase, DNA is duplicated. Once the duplication of DNA is done, cells move into G2 where the size of the cell increases and proteins to initiate mitosis are synthesized. In M, the cells undergo mitosis.

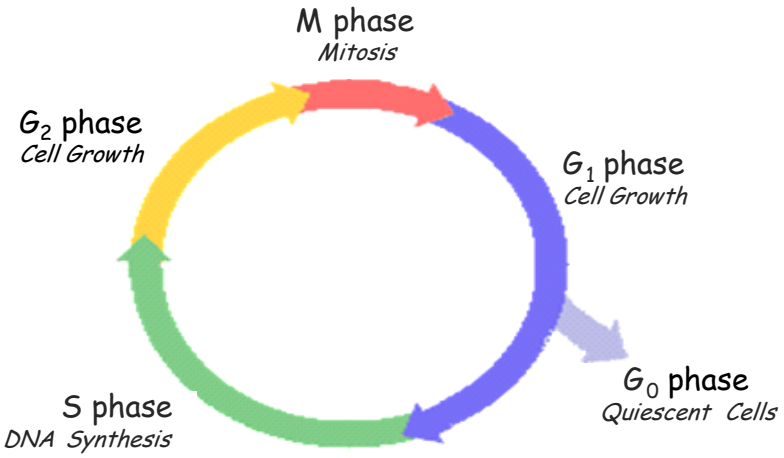


Figure 1.1 The Cell Cycle

Progression through the cell cycle in cells is regulated by two classes of molecules, cyclins and cyclin-dependent kinases (CDKs). The level of CDKs remains relatively constant throughout the cell cycle, while the level of cyclins varies from one phase of the cell cycle to another. Transit through the cell cycle occurs when cyclins bind to CDKs. For example, progression through G1 is moderated by cyclin D/CDK4/6, a complex that phosphorylates the retinoblastoma tumor suppressor protein which is an important regulator of cell cycle progression through G1 to S, while progression through S is moderated by cyclin A/CDK2, a complex that regulates the initiation and progression of DNA synthesis, and finally, progression through the G_2 phase is moderated by cyclin B/CDK1, a complex that induces mitosis by phosphorylating and activating enzymes regulating chromatin condensation, nuclear membrane

breakdown and microtubule reorganization. A diagram of the cyclin/CDK complexes and where they act within the cell cycle is shown below in Figure 1.2.

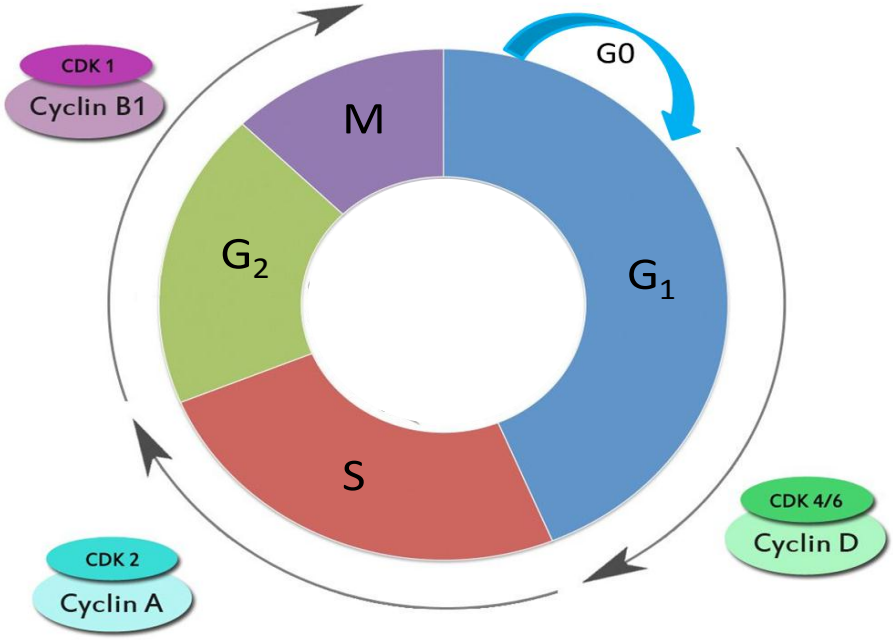


Figure 1.2 Cell Cycle Progression

Advancement through the cell cycle requires cells to pass through three cell cycle checkpoints, the G_1/G_0 checkpoint, the S checkpoint, and the G_2/M checkpoint. Halting progression at these checkpoints (cell cycle block) allows the cell to repair DNA damage before progressing to the next phase of the cell cycle. At the G_1/G_0 checkpoint, a decision is made for the cells to divide, delay division or enter a resting phase. The S phase checkpoint involves verification of the DNA synthesis process. If errors are found when replicating the DNA, the cell will not progress all the way through the S phase. The third checkpoint, G_2/M, is a point in the cell cycle where, if any DNA damage is detected, the cell will not enter mitosis and divide. The cell cycle checkpoints are shown in Figure 1.3.

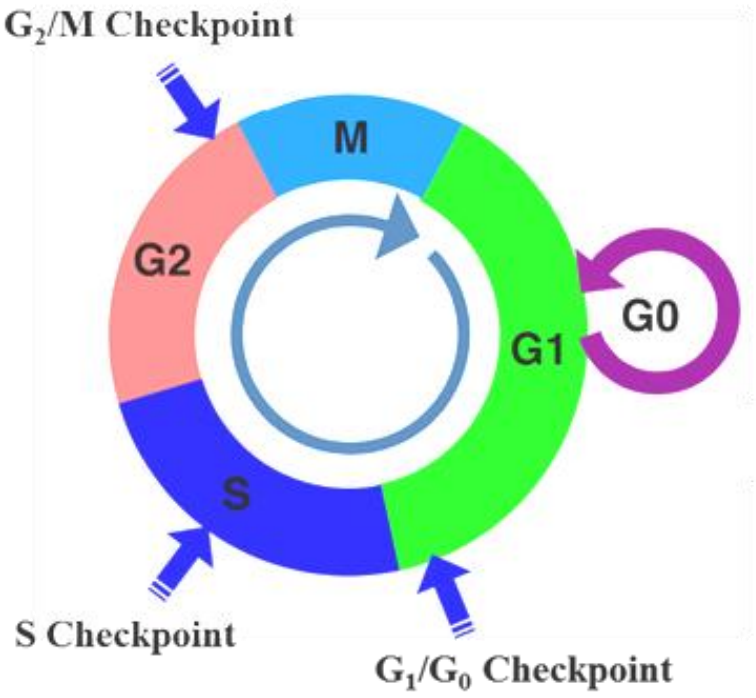


Figure 1.3 Cell Cycle Checkpoints

1.6.2 Apoptosis

Another mechanism that cells have developed to cope with damaging agents, such as ionizing radiation, is apoptosis, or programmed cell death. Apoptosis is characterized by morphological changes such as chromatin condensation, cell shrinkage, nuclear and cytoplasmic blebbing, and formation of membrane bound apoptotic bodies. Apoptosis occurs throughout development, aging, and as a response to cellular damage caused by radiation or other chemotherapeutic agents such as genistein. There are two main pathways through which apoptosis occurs: the extrinsic pathway (death receptor pathway) and the intrinsic pathway (mitochondrial pathway).

The extrinsic signaling pathways initiating apoptosis involve death receptors that are the members of the tumor necrosis factor (TNF) receptor gene superfamily [112]. Members of the TNF receptor family from a cytoplasmic domain of approximately 80 amino acids termed the

"death domain," which play a critical role in transmitting the death signal from the cell surface to the intracellular pathways [113]. A death-inducing signaling complex (DISC) is formed that cleaves caspase 8, thus activating it. Once activated, caspase 8 is capable of activating an effector protein which initiates degradation of the cell.

The second apoptotic pathway, the intrinsic pathway, is initiated through the mitochondrion when there is cellular stress. Upon receiving the stress signal, proapoptotic proteins in the cytoplasm and apoptotic signal molecules bind to the outer membrane of the mitochondriom to trigger release of cytochrome *c*. Following its release, cyctochrome c forms a complex in the cytoplasm with ATP and Apaf-1. After the formation of the this complex, caspase 9 is activated that works together with the complex of cytochrome c, ATP, and Apaf-1 to form an apoptosome which activates an effector protein that initiates degradation. Figure 1.4 shows the apoptotic pathways and the proteins involved in activating apoptosis.

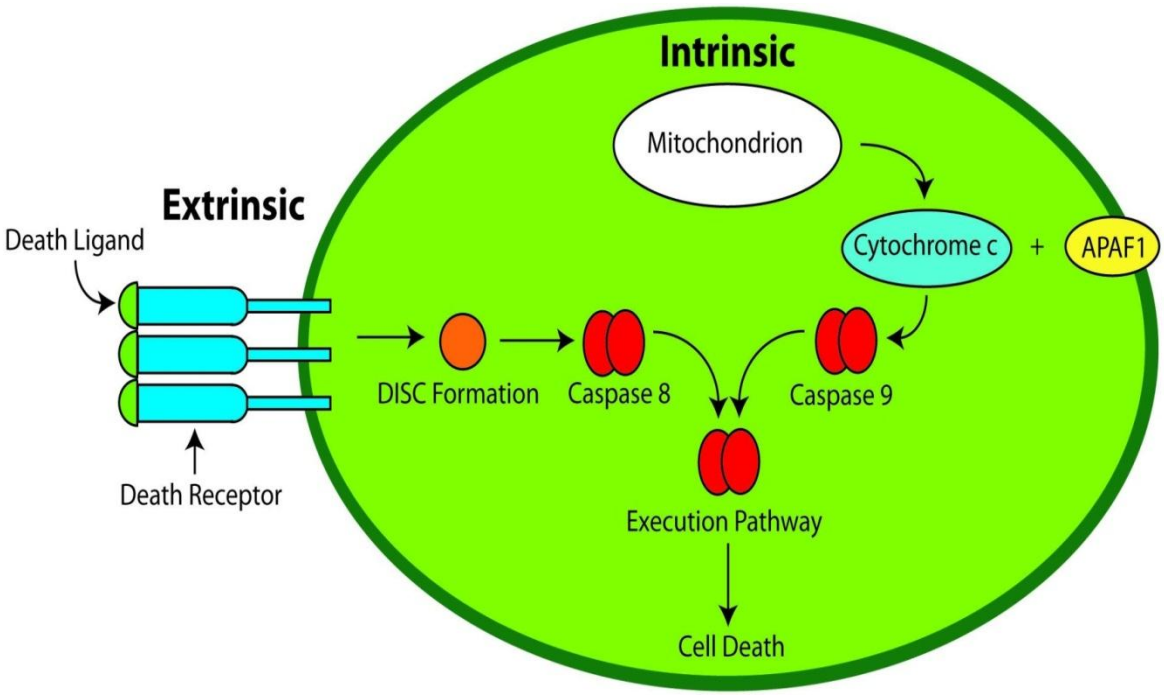


Figure 1.4 Intrinsic and extrinsic pathways of apoptosis.

1.7 HYPOTHESIS AND SPECIFIC AIMS

Exposure to radiation, especially low doses of radiation, is increasingly becoming a worldwide health issue. Because of this, the need for effective radioprotectors that can attenuate or mitigate radiation damage is critical. Unfortunately, amifostine, which acts by scavenging free radicals that may be created when ionizing radiation interacts with the cells of the body, is currently the only radioprotector that is specifically approved by the Food and Drug Administration for use as a radioprotector. However, amisfostine is limited in its impact due to its cytotoxicity at doses necessary for radioprotection. Furthermore, it must be present during radiation exposure, since a delay in administering it negates its effectiveness.

As stated earlier, characteristics of an effective radioprotector are that the compound not only possesses strong radioprotective ability, but that it must be readily available, relatively non-toxic and easily administered. It is the hypothesis of this dissertation that fermented soy miso meets these requirements. Since the first written record in 700 BC [83] within cultures who have used miso as a food, it has been clearly understood that miso possesses remarkable health-promoting properties. Miso has been credited with a host of beneficial effects, among which are its radioprotective and antioncogenic properties. Unfortunately, few rigorous studies of miso's reputed actions have been undertaken to date. It is the objective of the specific aims set forth below, therefore, to better identify and characterize the radioprotective and antioncogenic properties of miso and to compare its effects with those of genistein (miso's most abundant active ingredient), using two experimental prostate cell lines, one representing a tumorigenic cell population (PC3 cells) and one representing a non-tumorigenic "normal" cell population (RWPE-1 cells). To accomplish this, the following specific aims have been developed.

1.7.1 Specific Aim 1

The first goal of this study is to further understand the effects of soybean miso and the most abundant active ingredient, genistein, on human prostate cell lines. Studies will be initiated to investigate cell survival following a series of radiation doses ranging from 0 – 100 cGy (0, 10, 50, and 100 cGy), with survival ascertained using the standard clonogenic assay [114]. The experiments will include comparing and contrasting the radioprotective and antioncogenic properties of soy miso and genistein in the tumorigenic, human prostate carcinoma PC3 cells and in the non-tumorigenic, human epithelial prostate RWPE-1cells.

1.7.2 Specific Aim 2

The second goal is to identify the effects of radiation, miso, and genistein on the cell cycle. A major defense against the effects of ionizing radiation is cell cycle arrest or "block". Genistein, one of the active ingredients in miso, is also reported to cause cell cycle block [100, 101, 103, 108]. The experimental design will be similar to that for the first specific aim in that this aim will be comparing and contrasting the radiomodulating effects of soy miso and genistein treatment. Irradiation will be with doses ranging from 0 – 100 cGy on the two experimental models chosen to represent tumor and "normal" prostate cells. Detection of potential cell cycle blocks at the various checkpoints (G_0/G_1, S, G_2/M) will be accomplished using Western Blot analysis for key cell cycle proteins (cyclins D, A and B, respectively).

1.7.3 Specific Aim 3

The third goal is to investigate the effect that genistein (10 μM and 30 μM) and soy miso have on the apoptotic response to radiation in tumorigenic and non-tumorigenic prostate cells.

Another major response to the effects of ionizing radiation in cells can be the induction of apoptosis. Additionally, genistein, one of the active ingredients in miso, is reported to induce apoptosis in tumorigenic cell lines, but not in non-tumorigenic cells [115]. To meet this aim, studies will be initiated that compare and contrast the radiomodulating effects of genistein and miso on the PC3 and RWPE-1 cell lines. Both the extrinsic and intrinsic pathways of apoptotic signaling will be monitored following radiation doses from 0-100 cGy by Western Blot analysis using activation of caspase 8 (extrinsic pathway indicator) and caspase 9 (intrinsic pathway indicator).

CHAPTER 2: MATERIALS AND METHODS

2. 1 ANTIBODIES AND REAGENTS

Primary antibodies cyclin A, cyclin B, cyclin D, caspase 8, caspase 9, cleaved caspase 8, and cleaved caspase 9 were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies (anti-mouse AP-conjugated and anti-rabbit AP-conjugated) were also purchased from Cell Signaling Technology (Danvers, MA). Genistein was purchased from LKT Laboratories, Inc. (St. Paul, MN). Fetal calf serum was purchased from HyClone Laboratories (Logan, UT). Iscove's Modified Dulbecco's Media and penicillin/streptomycin were purchased from Sigma Aldrich (St. Louis, MO). Keratinocyte Serum Free Media, Bovine Pituitary Extract, and Epidermal Growth Factor were purchased from Life Technologies (Grand Island, NY). The chromogenic substrate for the Western blots (nitro blue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) was purchased from Promega (Madison, WI).

2. 2 TUMOR CELL LINES AND CULTURE CONDITIONS

The experimental models for this study are two prostate cell lines which represent the opposite ends of the spectrum with regard to tumorigeniciy – human RWPE-1 prostate epithelial cells (non-tumorigenic model) and human PC3 prostate carcinoma cells (tumorigenic model). Both of these cell lines are well established lines that have been used in several laboratories and numerous investigations. Thus, a strong data base exists upon which to obtain relevant information pertaining to cellular response, culture conditions, and growth characteristics. Briefly, the PC3 cell line was established from metastatic tumor tissue in a lumbar vertebra of a 62-year-old man diagnosed with poorly differentiated prostatic adenocarcinoma [116]. PC3 cells are androgen insensitive and have a high metastatic potential. They are often used as a model for

prostate cancer in both experimental cell and animal studies. In contrast, the RWPE-1 cell line was established from a histologically normal prostate of a 54-year-old man undergoing a cystoprostatectomy [117]. After removal from the prostate, these cells were immortalized using a human papilloma virus (HPV 18). Although immortalized, RWPE-1 cells exhibit normal epithelial morphology and do not possess tumorigenic potential as evidenced by their failure to produce tumors when injected into nude mice.

Both cell lines were obtained from American Type Tissue Culture (ATCC). RWPE-1 cells were routinely maintained under sterile conditions in Keratinocyte Serum Free Medium supplemented with 0.05 mg/ml of bovine pituitary extract and 5 ng/ml of human recombinant epidermal growth factor, while PC3 cells were maintained under sterile conditions in Iscove's Modified Dulbecco's Media supplemented with 10% fetal calf serum. Both cell lines were incubated at 37°C, 5% CO_2, and 95% air. To avoid overgrowth, cell cultures were passaged weekly by trypsinization and replating at a density of 5000 cells per cm^2.

2.3 GENISTEIN PREPARATION

Genistein powder (LKT Laboratories, Inc, St. Paul, MN) was dissolved in dimethyl sulfoxide (DMSO) to make a 5 mmol/L solution. Cells were placed in 35 mm Petri dishes with 1.5 ml of culture media. The two doses of genistein used in this investigation were 10 μM and 30 μM. These doses were obtained from the literature and represent two frequently studied doses used in investigating the radiomodulating effects of genistein on prostate cells [94, 95, 110, 111, 118-121]. For the 30 μM concentration, 12 μl of the genistein mixture was added to the dish and then 488 μl of culture media was added to yield a final volume of 2 ml. For the 10μM

concentration, 4 μl of the genistein mixture was added to the Petri dish along with 496 μl of culture medium to yield a final volume of 2 ml.

2.4 MISO PREPARATION AND DETERMINATION OF MISO DOSE

America's First Organic Light Miso, Miso Master Mellow White Organic Traditional Soy Paste (American Miso Company, Ashville, NC), was provided by Dr. C. Ruth Kempf. The paste was diluted using sterile conditions as follows. Five grams of miso paste was measured out and then mixed with 50 ml of growth medium (IMDM for PC3 cells and KSFM for RWPE-1 cells) to a consistency of a thick soup.

Because no data on the radiomodulating effects of miso on prostate cell survival were available in the literature, experiments were designed using the clonogenic cell survival assay to obtain miso doses that were biologically equivalent to the two genistein doses being studied. Dose response data from the PC3 prostate cell survival studies are plotted in Figure 2.1. For ease of comparison, clonogenic cell survival in PC3 cells treated with 10 μM and 30μM genistein are also displayed. As can be seen, data demonstrate that a 1:1,000,000 dilution of miso produced a similar survival response to 10μM genistein and a 1:10,000 dilution of miso produced a similar survival response to 30μM genistein. When tested on the non-tumorigenic RWPE-1 cells, these miso dilutions were also found to be statistically equivalent to 10 μM and 30μM genistein concentrations (97±12 vs. 99±6 percent of untreated control for the low doses of genistein and miso, respectively, and 59±7 vs. 62±10 percent of untreated control for the high doses of genistein and miso, respectively).

Determination of Miso Dose Using the Clonogenic Cell Survival Assay in PC3 Prostate Carcinoma Cells

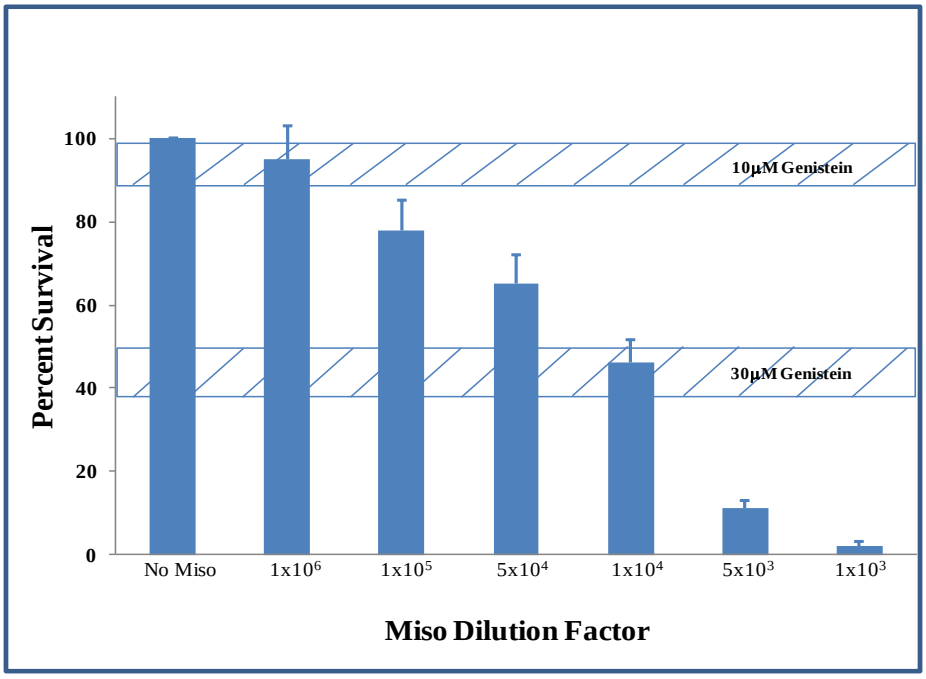


Figure 2.1 Determination of miso dose using the clonogenic survival assay in PC3 cells. Cells were treated with miso dilutions ranging from 1:1,000 – 1:1,000,000 and overall survival was assessed. Three independent experiments using triplicate dishes (n=9) for each data point were performed with the means and the SEM shown. The cross-hatched areas show 10μM genistein (mean ±SEM) and 30μM genistein (mean ±SEM) survival rates. Data demonstrate that a 1:1,000,000 dilution of miso produced a similar survival response as 10μM genistein and a 1:10,000 dilution of miso produced a similar survival response as 30μM genistein. As described in the text, similar results were found when using the RWPE-1 cell model.

2.5 TRANSWELL CULTURE EXPERIMENTS

Transwell Plate System - Soy miso is a "live" food product that is made by fermenting soybeans with koji mold (usually an *Aspergillus* variant). As a consequence, it contains both fungal and bacterial species. To prevent bacterial contamination during co-culture with cells, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin was added to the growth medium. However, to prevent fungal contamination of the experimental cell cultures, it was necessary to

prevent direct contact between miso and the human cell lines, but still allow humoral communication from this "live" food to occur readily via diffusion of macromolecular growth mediators. To accomplish this, the transwell plate system diagramed below in Figure 2.2 was used. This system uses tissue culture inserts containing a mesh-like membrane to physically separate the upper chamber where the miso is placed from the lower chamber that contains the growing cell cultures. However, the porosity of the membrane insert assures that any chemical mediators released from the miso can readily diffuse through the membrane and interact with cells in the lower chamber.

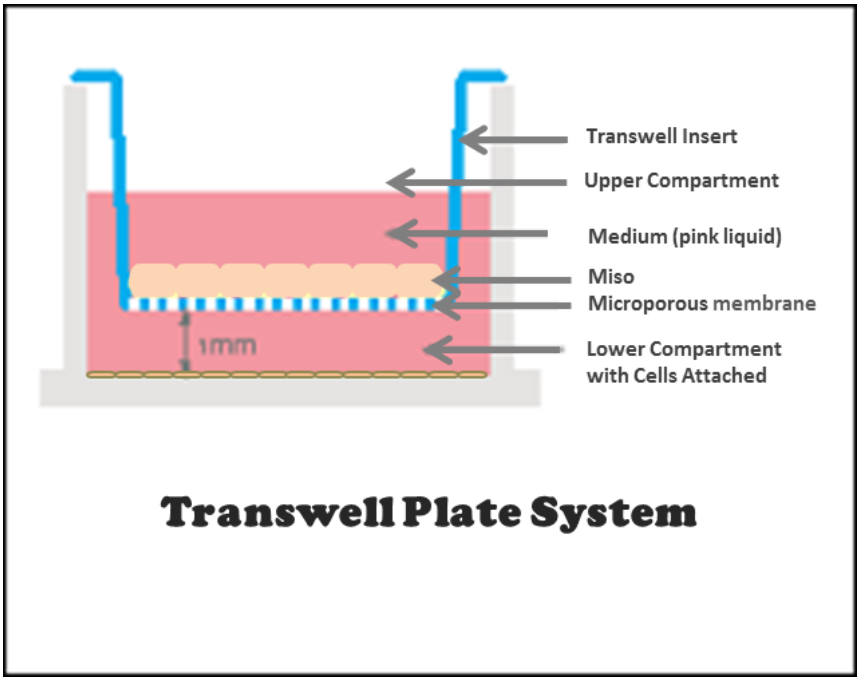


Figure 2.2 Side view of a transwell dish

Transwell Plate Protocol - Six-well transwell plates, with a 0.4µm porous membrane which was sufficient in these studies, were used when either the PC3 or RWPE-1 cells were treated with miso to obtain physical separation, but retain the ability for humoral communication. For all experiments, the transwell dishes were used under sterile culture conditions, and cells

were placed in the lower chamber in 1.5 ml of culture medium. The porous insert was then carefully inserted and 200 µl of miso at the appropriate dilution was added to the top of the membrane along with 300 µl of culture medium. In the case of the control (non-miso treated cells), the same procedure occurred however 500µl of growth medium was placed on the top of the transwell insert. Transwell cultures were maintained in an incubator at 37°C and 5% CO_2 for seven days before enumeration of colony numbers.

2.6 IONIZING RADIATION

Basics of X-Ray Production

X-rays are produced through two different processes, bremsstrahlung and characteristic x-rays. Bremsstrahlung, or braking radiation, is the result of a radiative interaction between a high-speed electron and a nucleus [1]. The electrons are slowed or stopped when passing by a nucleus of an atom, in particular a high atomic number material such as the tungsten target used in an x-ray tube. The loss of kinetic energy caused by the deceleration of the electron is radiated in the form of an x-ray, called a bremsstrahlung x-ray. Because the energy loss of the electrons can vary, bremsstrahlung has a continuous distribution with the maximum energy equal to the peak kilovoltage (kVp) used [5].

Characteristic x-rays are produced when an electron interacts with the atoms in the target causing an orbital electron to be ejected, ionizing the atom. A vacancy is created and an outer orbital electron will fill this vacancy. After an outer electron fills the vacancy, the remaining energy (subtracting out the binding energy) is emitted as a characteristic x-ray with a discrete energy [1]. The x-rays are called characteristic because the energy is defined by the atoms in the target and the electron shells involved [5].

X-rays are typically presented as a spectrum. This spectrum is a combination of both bremsstrahlung and characteristic radiation, with some of the lower energy x-rays being filtered out naturally by the insulating oil surrounding the x-ray tube, the glass envelope of the x-ray tube, and the exit window [5]. Additional filtration is often added to the beam to filter out the lower energy x-rays, allowing the higher energy x-rays, which are more penetrating, to pass. The maximum energy of the x-rays produced is equal to the maximum potential applied across the x-ray tube.

X-ray Source - The Siemens Stabilipan x-ray generator located in Dr. Roberta Johnke's laboratory within the Ed Warren Life Sciences Building at the East Carolina University Brody School of Medicine campus was chosen for use in this study due to its accessibility, the uniformity of dose distribution when irradiating cells, and the availability of literature in which to compare the data observed in this study. The energy used in this study (250 kVp) was chosen because the x-rays are energetic enough to pass through the samples without significant attenuation (i.e. across the size of cells, the ratio of the initial intensity to the final intensity is 0.9998). This value was calculated according to the following equation and using a phantom cell model that was 100% water and a cell height of 10μm that was irradiated at 250 kVp:

$$\frac{I}{I_0} = e^{-\mu x}$$

This energy range is also important when calculating the Relative Biological Effect (RBE) because the ratio is calculated by comparing one type of radiation source to 250 kVp x-rays.

Briefly, in a stationary anode x-ray tube such as that in the Siemens Stabilipan, the source of electrons is a filament that is heated and emits electrons in a process called thermionic emission [1]. The electrons are accelerated toward a tungsten target by application of a high potential difference between the cathode (the filament) and the anode (the target). The quantity

of electron flow (current) in the x-ray tube is described in units of milliamperes (mA) [5]. The voltage (kVp) primarily determines the maximum x-ray energy produced. The total number of x-rays produced at a set kVp depends directly on the product of the mA and exposure time, and is typically described in terms of mA-s or mAs [1].

For the Siemens Stabilipan used in this dissertation, the continuous x-ray spectrum had a minimum energy of approximately 50 kV and maximum energy of 250 kV. The major component of the x-ray spectrum was from bremsstrahlung radiation although a minor contribution to the x-ray spectrum occurs at approximately 60 and 70 kV from the characteristic x-rays produced from the tungsten target.

Quality Assurance - Bi-monthly quality assurance procedures were conducted using a Victoreen Condenser R-Meter Ionization Chamber Model Number 570 (serial number 9887). The field uniformity of the orthovoltage beam was measured using Gafchromic® EBT2 film. After exposure, the film was scanned using an Epson Perfection v750 Pro Scanner and analysis was performed using ImageJ software. Uniformity was determined to have less than a 2% deviation from the mean output which is within clinical tolerances.

Radiation Procedure - Exponentially-growing cultures of RWPE-1 and PC3 cells were grown to 70-80% exponential phase and then treated with genistein, or miso for 16 hours. Treated cells were then exposed to varying doses of xRT from 0-100 cGy (0, 10, 50 and 100cGy). The set-up for the Siemens Stabilipan x-ray generator was 250kVp, 15mA, 100 cm site-to source distance (SSD), and 2mm Cu filtration. Dose rate was 16.6 cGy/min. A schematic of the radiation set-up is presented below in Figure 2.3.

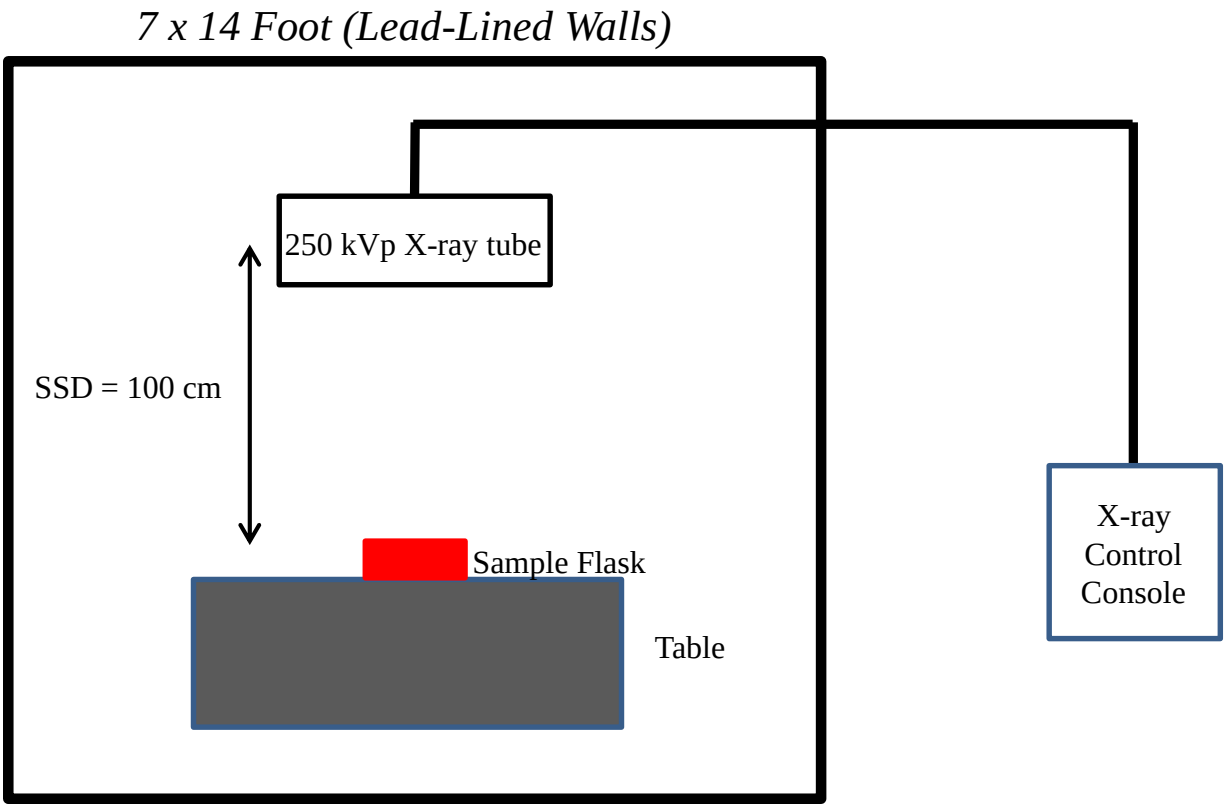


Figure 2.3 Schematic of x-ray suite

2.7 CLONOGENIC CELL SURVIVAL ASSAY

The fraction of cells that survive after irradiation was assessed by the clonogenic survival assay which measures the fraction of cells that continue to divide for at least five times after irradiation. For this assay, cells were grown to exponential phases, and harvested by trypsinization, counted on a hemocytometer and diluted to $1x10^4$ cells/ml of growth media. Cells were seeded in 35 mm petri dishes with 2 ml of growth medium with or without genistein (concentration of 10 μM or 30 μM). The number of cells added to each dish was chosen to yield 40-60 colonies per dish. Triplicate dishes were used for each experimental data point and at least three separate experiments were performed to obtain mean and standard error of the mean. Cells were allowed to settle and attach for 16 hours before being exposed to irradiation at various

doses. Dishes were then incubated at 37°C and 5% CO_2 in a humidified incubator for 8 hours. Following this time, dishes were removed from the incubator and the culture medium gently removed. New culture medium without genistein was gently added back to the dishes, and the dishes were placed into the incubator for 7 days. Colonies were then removed from the incubator, washed with PBS, air dried for two hours, stained for 6 minutes with 0.5% crystal violet in methanol, and washed with running tap water to remove nonspecific staining. Colonies (>50 cells) were enumerated under a Fisher Stereomaster II 10X stereoscopic microscope. For each triplicate set of dishes, the mean, the standard deviation, and standard error was calculated. The cell survival fraction (SF) was calculated from the formula:

$$\mathrm{SF} = \frac{\text{Number of Colonies per plate}}{\text{Number of cells seeded per plate}}$$

The procedures for assaying clonogenic survival of irradiated cells with and without miso was identical to those used for culturing survival of irradiated cells with and without genistein with the exception that 35 mm transwell dishes (see Figure 2.2 above) were used in place of regular 35 mm petri dishes to prevent fungal contamination of the cell cultures and the addition of antibiotics prevented bacterial contamination

.

2.8 WESTERN BLOTTING

The Western blot procedure is a widely used analytical technique to detect specific proteins within a cellular extract or tissue homogenate [122]. It uses gel electrophoresis to separate denatured proteins according to their polypeptide length. The proteins are then transferred to a PVDF membrane where they are detected by probing with antibodies specific to the target protein.

To obtain cell lysates for Western blot analysis, RWPE-1 and PC3 cells were grown to exponential phase (60-70% confluence), treated with genistein (10 μM or 30 μM) or miso for 16 hours prior to irradiation. Cells were irradiated at the appropriate dose (0, 10, 50, & 100 cGy) and incubated for either 2 or 5 hours prior to harvesting by trypsinization. Harvested cells were then pelleted at 400g for ten minutes, washed and resuspended in ice cold lysis buffer (25mM Tris, 50mM NaCl, 2% Nonidet, 0.2% SDS, and 0.5% deoxycholic acid, Halt Protease Inhibitor Cocktail at a pH of 7.4), and incubated for 15 minutes on ice with vortexing performed every 5 minutes. The cell extracts were then centrifuged (10 minutes, 10,000g, 4°C), supernatants (lysates) aliquoted into multiple tubes and stored at -20°C until analysis. Protein content for the lysates was determined using Bradford analysis. For Western blotting, lysates were mixed 1:1 with 2X sample loading buffer (100 mM Tris-HCL pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM dithiothreitol) and samples (20μg - 65μg depending on protein examined) were resolved on SDS-polyacrylamide gels at 100V constant voltage in standard buffers (25mM Tris with 200mM glycine and 0.1% SDS). Pre-stained molecular weight markers were run on gels to provide a visible quality control on protein transfer along with molecular weight standards (Santa Cruz, CA). After electrophoresis, gels were rinsed in ice cold transfer buffer (24.8 mM Tris base, 192mM glycine, 10% methanol, pH 8.3) and proteins transferred to polyvinyl difluoride (PVDF) membranes at 100V constant voltage for 1 hour. The resulting blots were incubated overnight at 4°C in blocking buffer, which was composed of 0.1% Tween-20 with 5% w/v nonfat dry milk in TBS or 0.1% Tween-20 with 5% bovine serum albumin depending on the protein being studied, then incubated with the primary antibody being studied diluted in TBST (TBS, 0.1% Tween-20) for 3 hours. The dilution for the cyclin B1 primary antibodies was 1:2000 while the dilution for cyclin A, cyclin D, caspase 8, cleaved caspase 8,

caspase 9, and cleaved caspase 9 was 1:1000. The PVDF membrane was then incubated for 2 hours with biotinylated secondary antibody conjugated with streptavidin-alkaline phosphatase anti-IgG (anti-mouse or anti-rabbit, Cell Signaling Technology, Danvers, MA). To visualize the bands, membranes were incubated with 10 ml of nitro blue-tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate as substrate (Promega, Madison, WI). Resultant bands were scanned and analyzed by ImageJ software (National Institute of Health).

2.9. STATISTICAL ANALYSIS

For all experiments the mean ($\bar{x}$) was calculated using the equation below. For the cell survival data n was equal 9 and for the Western blot data n was equal to 3.

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} n_i$$

The standard deviation (s) for all experiments, the standard deviation was calculated using the equation below using the same values of n mentioned above.

$$s = \sqrt{\left(\frac{1}{n-1}\right)\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

The standard error of the mean (SEM) was calculated using the equation below with n=9 for the cell survival data and n=3 for the Western blot data. All error bars shown in the figures in the Results section represent the SEM.

$$SEM = \frac{s}{\sqrt{n}}$$

Statistical analysis was performed to yield a test statistic, the p-value, which is the probability of obtaining a test statistic at least as extreme as the one that is actually observed assuming the null hypothesis is true. If the p-value was less than the predetermined value of 0.05, the data indicates that the observed result would be unlikely under the null hypothesis.

In this study, two data points were considered significantly different if the Student's t-test performed yielded a p-value ≤ 0.05. If more than two data points were being examined, a two-way analysis of variance (ANOVA) was performed. If the p-value was ≤ 0.05, the differences were deemed to be significant. The author would like to acknowledge the help and advice received from the Biostatistics Department in the College of Allied Health Sciences located at East Carolina University.

CHAPTER 3: RESULTS

3.1. LOW DOSE RADIATION CELL SURVIVAL RESPONSE IN PC3 AND RWPE-1 CELLS FOLLOWING TREATMENT WITH MISO OR GENISTEIN

The goal of this set of experiments was to characterize the radioprotective and antioncogenic abilities of both the fermented soy food miso and genistein, its most abundant active ingredient. To accomplish this goal, radiation cell survival was assessed over a range of low dose exposures following treatment with both high and low doses of either miso or genistein. As stated previously, the clonogenic cell survival assay was used to assess survival in these experiments. This procedure, which tests for a cell's ability to undergo unlimited division, was chosen because it has long been considered to be the "gold standard" for determining cell survival following radiation insult.

3.1.1 Lack of Radioprotection Following Treatment with Genistein or Miso

Figure 3.1 shows the cell survival curves of human prostate carcinoma PC3 cells (panel A) and human non-tumorigenic prostate epithelial RWPE-1 cells (panel B) over a dose range of 0 to 100 cGy. Data represent the mean ± SEM of three independent experiments and are expressed as a percent of untreated control. Because an adequate model does not exist for radiation doses below 1 Gy, the lines connecting the data points are for ease of visualization of the different treatment groups. As can be observed, for both PC3 and RWPE-1 cells, the absence of increased survival in the experimental miso and genistein treatment groups when compared to the xRT Only group suggests that no apparent radioprotection is occurring following administration of either high or low doses of miso and genistein. On the contrary, as demonstrated by the significant reduction in radiation cell survival in both cell lines, treatment with either high dose genistein (30μM) or high dose miso (1:10,000 dilution) results in a

pronounced growth inhibitory action. This growth inhibitory effect, however, appears to be concentration dependent, since the results from Figure 3.1 also show that the radiation cell survival curves following administration of low doses of either genistein (10μM) or miso (1:1,000,000 dilution) resulted in no significant deviation from the xRT Only group's survival response.

3.1.2 A Potential Synergistic Interaction May Be Occurring Following Combined Radiation and Genistein or Miso

As well as plotting survival as a function of untreated control (Figure 3.1), results were also expressed as a percent of each treatment group's own unirradiated control and presented in Figure 3.2. Displaying the data in this manner was done in an attempt to determine if the combined radiation and treatment effects were additive, less than additive or more than additive in nature. Briefly, if survival curves for a miso or genistein treatment group overlay the xRT Only curve, the effect of the treatment is most likely additive to the radiation response. If survival curves for a miso or genistein treatment group are above the xRT Only curve, then the effect of the treatment plus the radiation is less than additive, and suggests that the two treatments may be partially overlapping each other in their actions. Finally, if survival curves for a miso or genistein treatment group are below the xRT Only curve, then the effect of the treatment and radiation is more than additive, and suggests a synergistic interaction could be occurring. As can be observed in Figure 3.2, the radiation cell survival curves of PC3 cells (Panel A) treated with both high dose miso and high dose genistein fall well below the xRT Only curve, suggesting that combining either of these two treatments with radiation results in a more than additive (synergistic) response in this tumorigenic cell model. Similarly, combining either high dose miso or high dose genistein with radiation in the non-tumorigenic RWPE-1 cell model (Panel B) also results in a radiopotentiating effect, but to a much lesser extent than is seen for the

PC3 cells. Unlike the responses observed following the combination of radiation with high doses of genistein and miso, however, radiation cell survival curves for both the low dose miso and low dose genistein treatment groups essentially overlay the xRT Only survival curve in both PC3 (Panel A) and RWPE-1 (Panel B) cell lines, suggesting that no significant radiopotentiation is occurring at these low concentrations.

Elimination of the low dose miso and genistein treatments: Since no significant growth inhibitory effects were observed for either low dose miso or low dose genistein (see Figure 3.1) and no radiomodulating action was induced (Figure 3.2), it was decided to focus only on the radiomodulating abilities of the high dose miso (1:10,000 dilution) and high dose genistein (30μM). In light of this and for ease of expression, therefore, the high dose miso is hereafter simply termed "miso" treatment and the high dose genistein is termed "genistein" treatment.

3.1.3 Demonstration of Potential Antioncogenic Action of Miso and Genistein Treatments

As well as genistein and miso exhibiting significant growth inhibitory action which appears to act synergistically with radiation to reduce cell survival, the data also suggest that the radiomodulating effects of genistein and miso treatments may be differentially expressed in tumor vs. non-tumor cells. To better display this differential response, Figure 3.3 plots clonogenic survival data as a function cell type following xRT Only (Panel A), xRT + miso (Panel B), and xRT + genistein (Panel C). Data demonstrate that, following radiation alone (Panel A), there are only minor, insignificant survival differences observed between the two cell lines studied in this project. However, after treatment with either miso (Panel B) or genistein (Panel C), significant cell specific differences are seen in the radiation cell survival curves, with the results suggesting that there is a much greater growth inhibitory effect being exhibited in the tumorigenic PC3 cell model than in the non-tumorigenic RWPE-1 cell model. Specifically,

following radiation exposures of 0, 10, 50 and 100 cGy, PC3 cells treated with xRT + miso had cell survivals of 43%, 35%, 30% and 24% of untreated control, respectively, while RWPE-1 cell survival was reduced to only 62%, 58%, 50% and 44% of untreated control, respectively. Similarly, following xRT + genistein treatment, PC3 cell survival values were reduced to 39%, 31%, 27% and 24% of untreated control for 0, 10, 50 and 100 cGy exposures, respectively, while RWPE-1 cell survival was reduced to only 59%, 54%, 44% and 39% of untreated control, respectively. The significantly lower cell survival values observed in the tumorigenic PC3 cell model versus the non-tumorigenic RWPE-1 cell model suggests that both miso and genistein may have antioncogenic potential that could possibly be translated into the clinic to improve the therapeutic index of radiation treatments.

Radiation Survival Curves for PC3 and RWPE-1 Cells Expressed as a Percent of Untreated Control

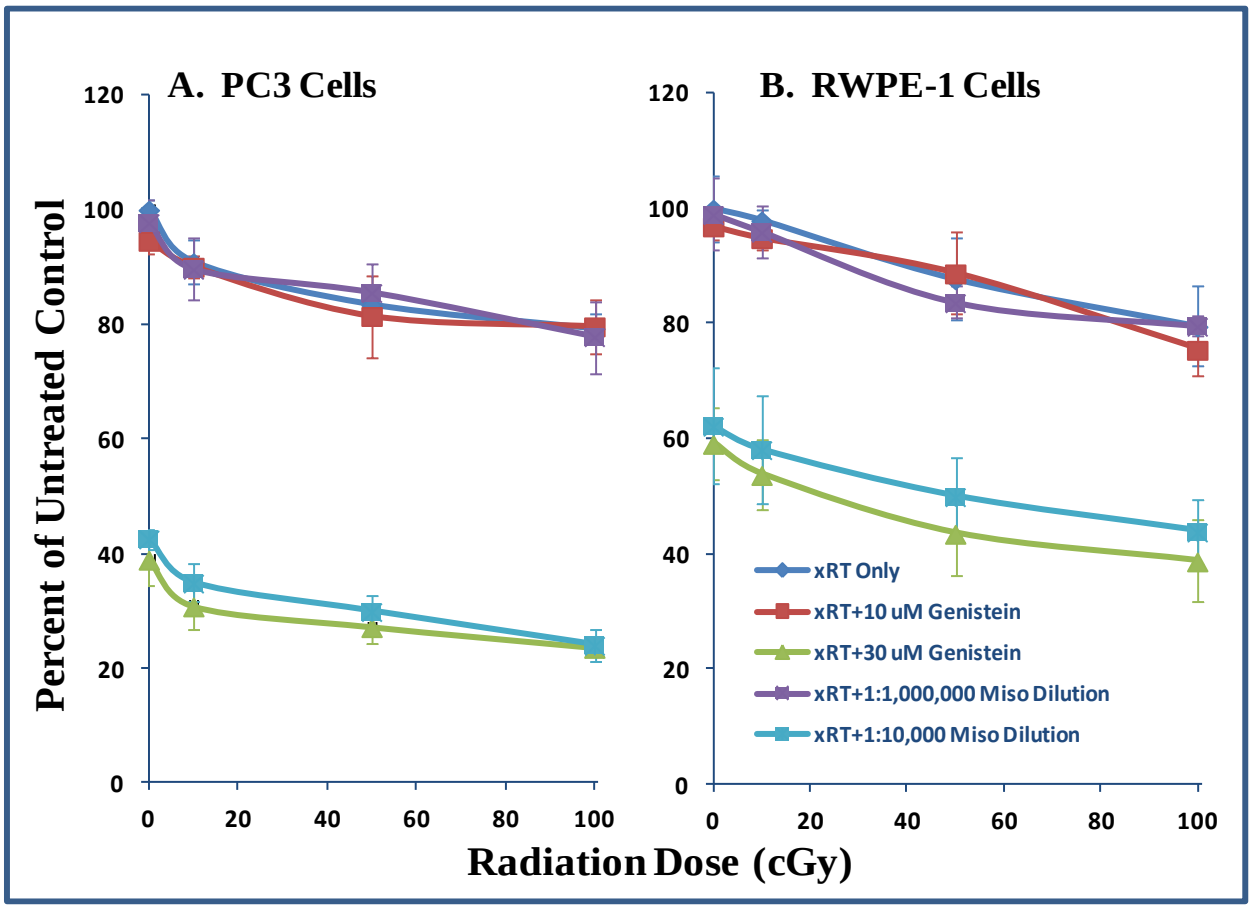


Figure 3.1 Cell survival curves of human prostate carcinoma PC3 cells (panel A) and human non-tumorigenic prostate epithelial RWPE-1 cells (panel B) expressed as a percent of untreated control (xRT Only). Data were obtained over a dose range of 0 to 100 cGy and represent the mean ± SEM of three independent experiments. For ease of visualization, points were connected with a line. Lack of enhanced survival in any of the treatment groups suggests that no radioprotection is occurring following administration of any of the experimental treatments. However, results do show that that a significant reduction in radiation cell survival was observed in both cell lines following treatment with high doses of either miso or genistein.

Radiation Survival Curves for PC3 and RWPE-1 Cells Expressed as a Percent of Treated Control

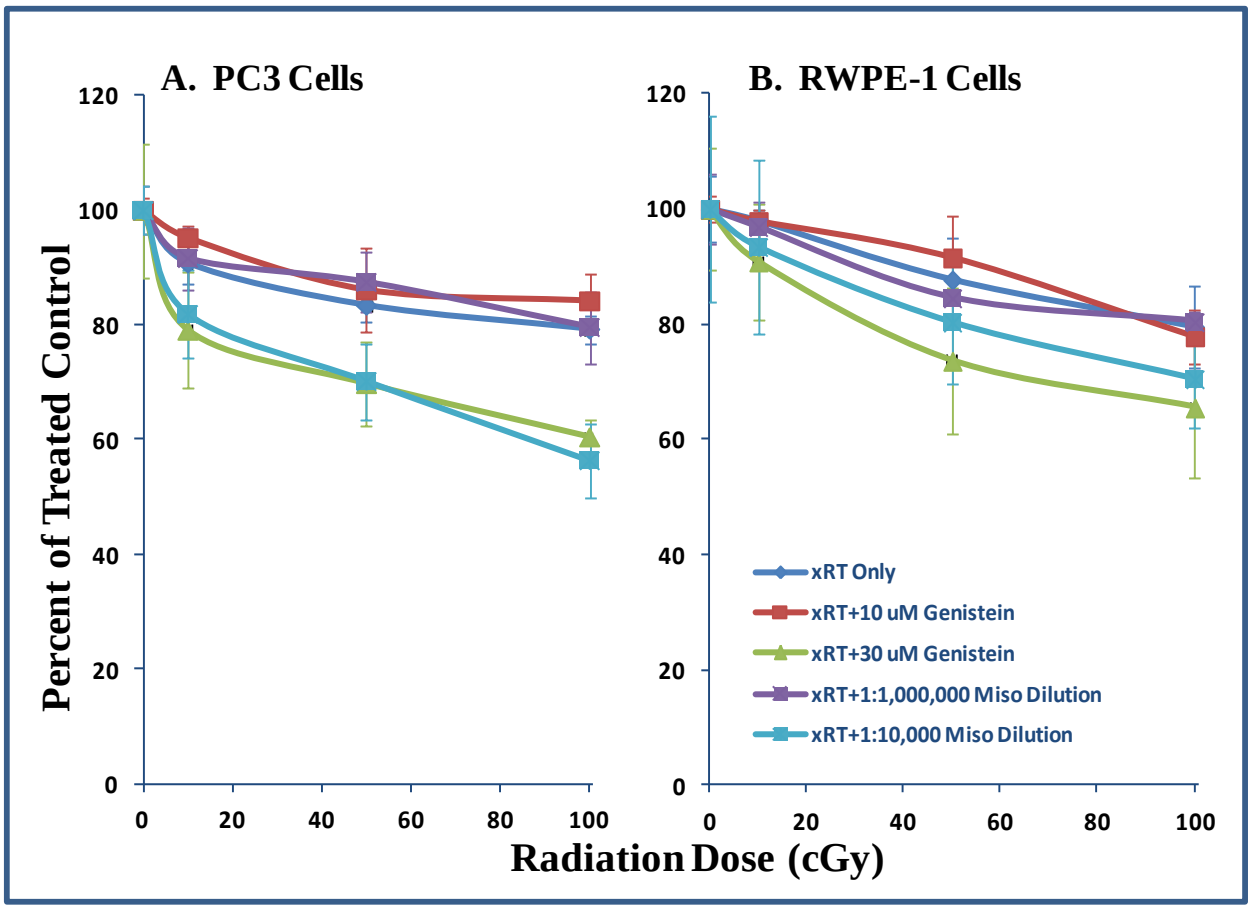


Figure 3.2 Cell survival curves of human prostate carcinoma PC3 cells (panel A) and human non-tumorigenic prostate epithelial RWPE-1 cells (panel B) expressed as a percent of control of each treatment group's own unirradiated control. Data were obtained over a dose range of 0 to100 cGy and represent the mean ± SEM of three independent experiments. For ease of visualization, points were connected with a line. Results show that combining radiation with high doses of either miso or genistein resulted in a more than additive reduction in cell survival in the PC3 tumorigenic model (Panel A), and, to a lesser extent, in the RWPE-1 non-tumorigenic model as well (Panel B).

Radiation Survival Curves for PC3 and RWPE-1 Cells Expressed as a Function of Cell Type

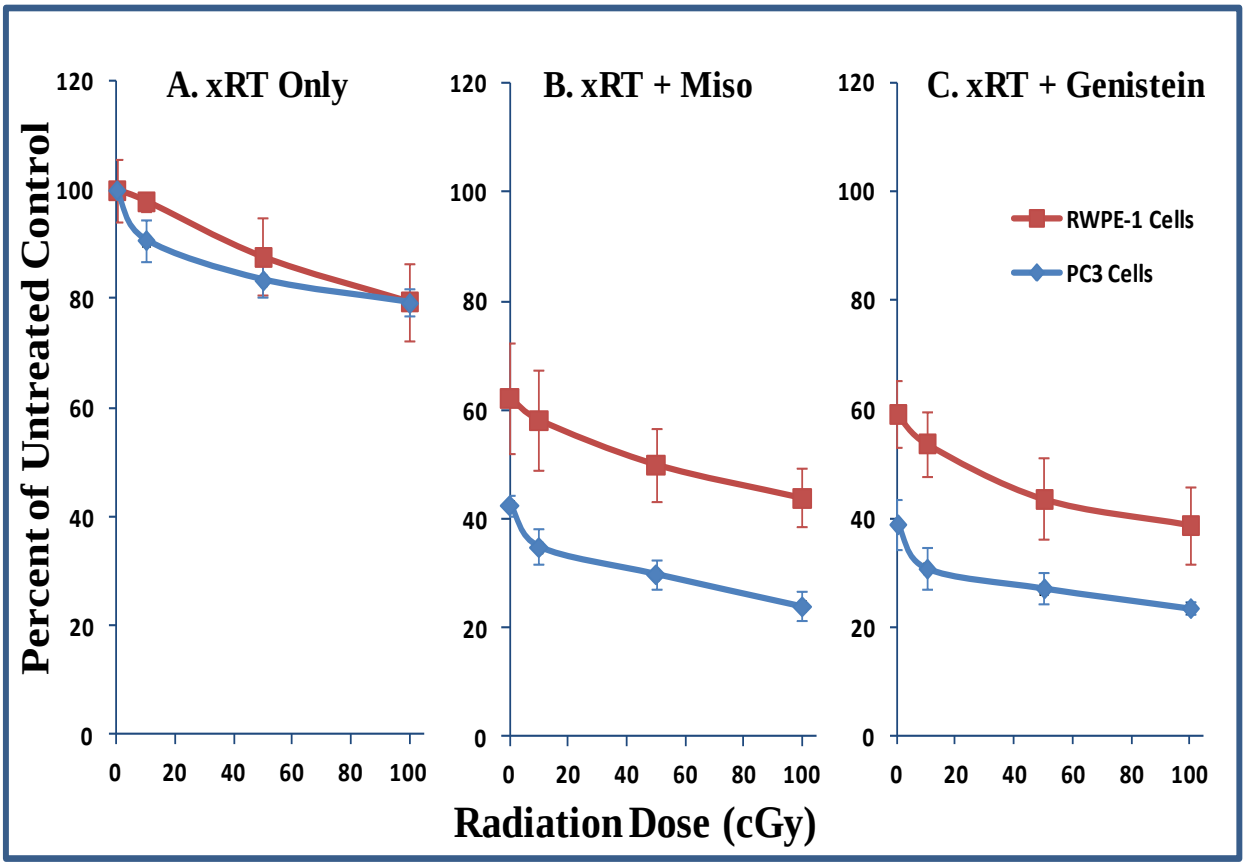


Figure 3.3: Comparison of clonogenic survival responses in PC3 cells (blue line) and RWPE-1 cells (red line) following radiation alone (Panel A), radiation plus miso (1:10,000 dilution), and radiation plus genistein (30µM). For ease of visualization, points were connected with a line. Data demonstrate significant cell line specific differences in radiation survival exist following treatment with genistein and miso. Results suggest that there is a much greater growth inhibitory effect on the tumorigenic PC3 cells than on the non-tumorigenic RWPE-1 cells.

3.2. CELL CYCLE CHANGES IN PC3 AND RWPE-1 CELLS AFTER LOW DOSE RADIATION AND TREATMENT WITH MISO OR GENISTEIN

Because it has been reported previously in the literature that both radiation and treatment with genistein may each cause a cell cycle block [100, 101, 103, 108], this set of experiments was designed to investigate whether one of the mechanisms underlying the growth inhibitory action of genistein and miso treatments involved the ability of these compounds to induce a cell cycle block, and, if a block is induced, at what phase in the cell cycle the block is occurring. The three cyclin proteins chosen to assay the various phases of the cell cycle were: cyclin D1 (*measured as an indicator of a block in G_0/G_1 phase*); cyclin A (*measured as an indicator of a block in the S phase*), and cyclin B1 (*measured as an indicator of a block in the G_2/M phase*). For all treatment groups, assay of cyclin changes were performed at both an early time point (2 hours post irradiation) and a late time point (5 hours post irradiation) in the cell's signaling response to radiation so as to ensure not missing any changes occurring.

3.2.1. Changes in Cyclin B1 Levels for PC3 and RWPE-1 Cells

xRT Only: Figure 3.4 displays the changes in cyclin B1 levels after exposure to low dose radiation alone for PC3 (Panel A) and RWPE-1 (Panel B) cells. Results are expressed as a percent of untreated control (± SEM; n=3) and represent the values obtained from cells harvested at both an early time point (2 hours post irradiation) and a late time point (5 hours post irradiation) in the cell's signaling response to radiation. The top panels display the bands of protein from representative Western blots, while the bottom panels represent the relative band intensities as analyzed by Image J software. As can be seen, at 100 cGy exposures, both cell lines display a significant decrease in cyclin B1 protein, indicating a G2/M phase block is occurring. However, results show only minor fluctuations in band intensity over the range of

radiation doses between 0 and 50 cGy, indicating that no G_2/M phase arrest is occurring at these radiation exposures.

xRT + Miso: Changes in the relative levels of cyclin B1 protein after treatment with xRT + Miso for PC3 cells (panel A) and for RWPE-1 cells (panel B) are shown in Figure 3.5. As above, results are expressed as a percent of untreated control (± SEM; n=3) and represent the values obtained from cells harvested at both an early (2 hours) and a late time point (5 hours) in the cell's signaling response to radiation. As in Figure 3.4, the top panels display the bands of protein from representative Western blots, while the bottom panels represent the relative band intensities as analyzed by Image J software. As can be observed, for both the tumorigenic PC3 cell model and the non-tumorigenic RWPE-1 cell model, data demonstrate a significant decrease in cyclin B1 levels at both early and late time points monitored when compared to untreated control (green bar), suggesting that a G_2/M block is occurring for both cell lines in this experimental treatment group. Furthermore, the observation that the reduction is present even when radiation is not given (0 cGy) suggests that the cyclin B1 response is a function of the miso treatment alone in this dose range, with little-to-no influence being derived from the radiation stress.

xRT + Genistein: Changes in the relative levels of cyclin B1 protein after treatment with xRT + Genistein for PC3 cells (panel A) and for RWPE-1 cells (panel B) are shown in Figure 3.6. As in Figures 3.4 and 3.5, results are expressed as a percent of untreated control (± SEM; n=3) and represent the values obtained from cells harvested at both an early and a late time point in the cell's signaling response to radiation. The top panels display the bands of protein from representative Western blots, while the bottom panels represent the relative band intensities as analyzed by Image J software. For both the tumorigenic PC3 cell model and the non-

tumorigenic RWPE-1 cell model, data demonstrate an almost identical response to genistein treatment as they do to miso (Figure 3.4) in that a significant decrease in cyclin B1 levels at both early and late time points is observed when compared to untreated control (green bar), suggesting that a G_2/M block is occurring for both cell lines in the genistein experimental treatment group. Also similar to the miso treatment group (Figure 3.5), over the 0-50 cGy dose range, the cyclin B1 reduction appears to be due to the genistein treatment alone and not a function of the radiation stress.

Summary of Cyclin B1 responses: For ease of comparison, Figure 3.7 summarizes the relative cyclin B1 low dose radiation responses (0-100cGy) in both the tumorigenic PC3 cells (panel A) and the non-tumorigenic RWPE-1 cells (panel B) for all three treatment groups at both time points monitored. Data indicate that little difference is observed in cyclin B1 levels as a function of the time after irradiation at which this cyclin is measured (2 vs. 5 hours post irradiation). Further, while higher doses of radiation (100cGy and above) are well known to induce a G_2/M cell cycle block [100, 101, 103, 108, 123], in these experiments, the lack of cyclin B1 reduction in the xRT Only group between 0-50 cGy suggests that these low dose exposures do not appear to be halting progression through this phase of the cell cycle. Finally, and of significance, results demonstrate that, for both the radiation + miso and radiation + genistein experimental groups, a marked reduction in cyclin B1 levels is seen at both early and late time points over the whole spectrum of radiation doses given, suggesting that a G_2/M phase cell cycle block is occurring as a function of the miso or genistein treatment and independent of the radiation-induced arrest except, perhaps, at 100 cGy. This block may be, at least in part, responsible for both the reduced cell survival seen following treatment with these compounds (Figure 3.1) and the potential synergistic growth inhibition observed when either of these

treatments is combined with low dose radiation (Figure 3.2). However, the lack of significant differences in the magnitude of cyclin B1 reduction between the PC3 and the RWPE-1 cell lines following either xRT + miso or xRT + genistein suggests that the antioncogenic action of these compounds is most likely occurring through some other mechanism.

Relative Cyclin B1 Levels in PC3 and RWPE-1 Cells Following xRT Only

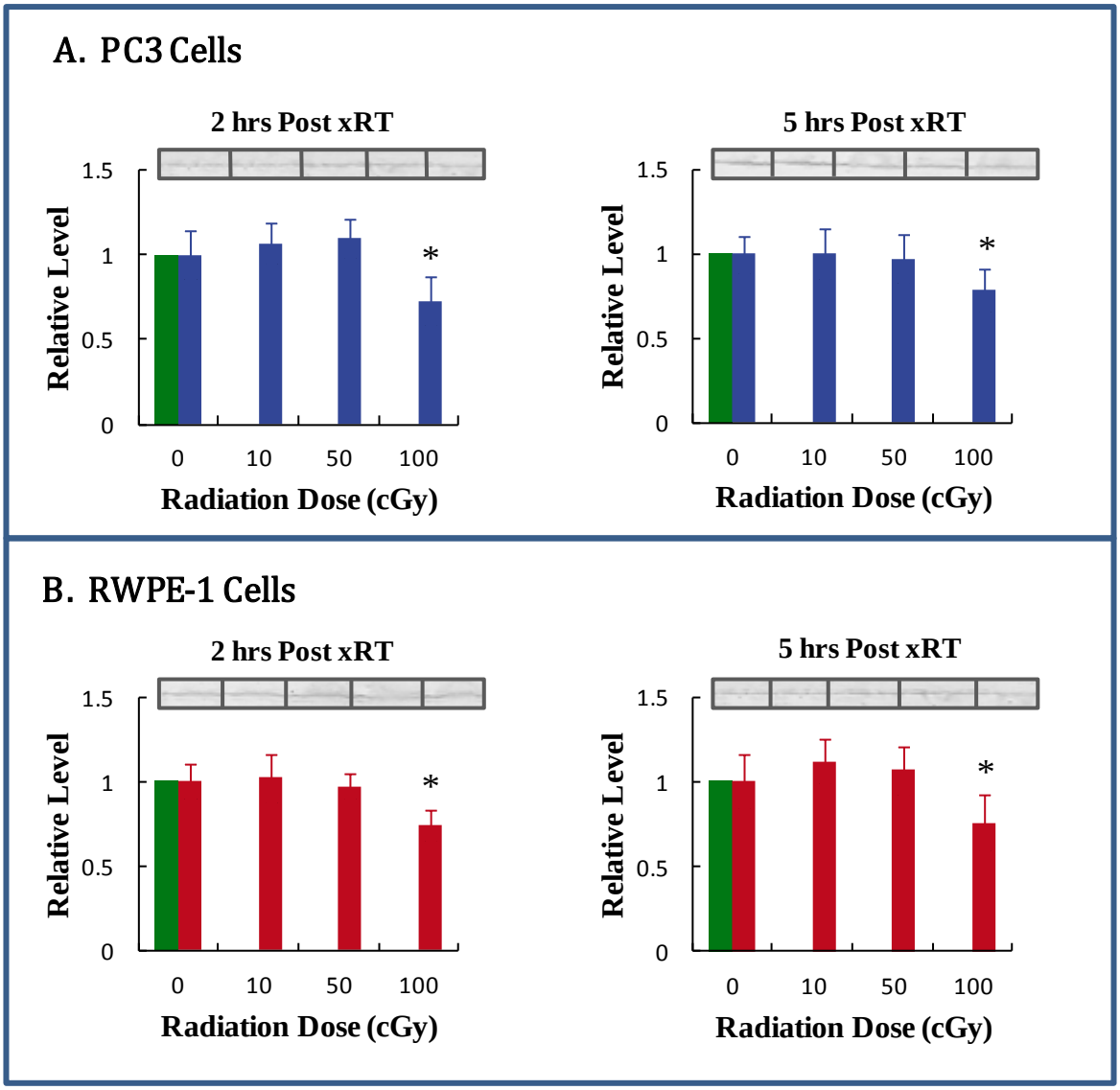


Figure 3.4 Changes in the relative levels of cyclin B1 protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following irradiation with doses ranging from 0-100 cGy. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. The bands of protein in a representative Western blot are displayed above each graph. The presence of a significant reduction in cyclin B1 protein in both the PC3 and RWPE-1 cell models at 100 cGy (indicated by an asterisk) suggest G_2/M phase arrest is occurring.

Relative Cyclin B1 Levels in PC3 and RWPE-1 Cells Following xRT + Miso

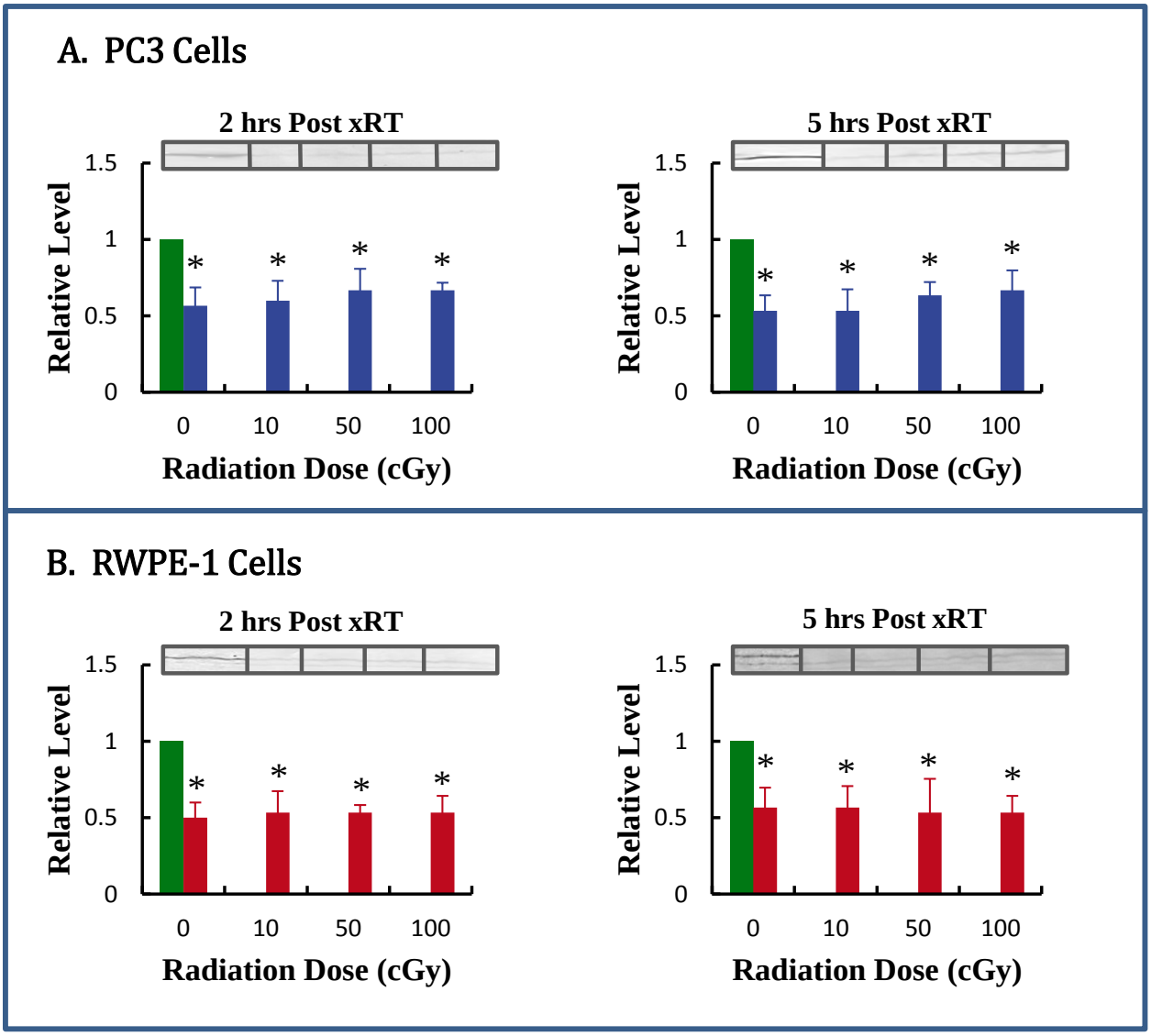


Figure 3.5 Changes in the relative levels of cyclin B1 protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following treatment with xRT + miso. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments were performed (with three different samples for each data point) with the means shown and the error bars representing the SEM. Representative Western blot bands of protein are displayed above each graph. Data demonstrate a significant reduction in cyclin B1 levels (indicated by an asterisk) when compared to untreated control for both PC3 and RWPE-1 cells.

Relative Cyclin B1 Levels in PC3 and RWPE-1 Cells Following xRT + Genistein

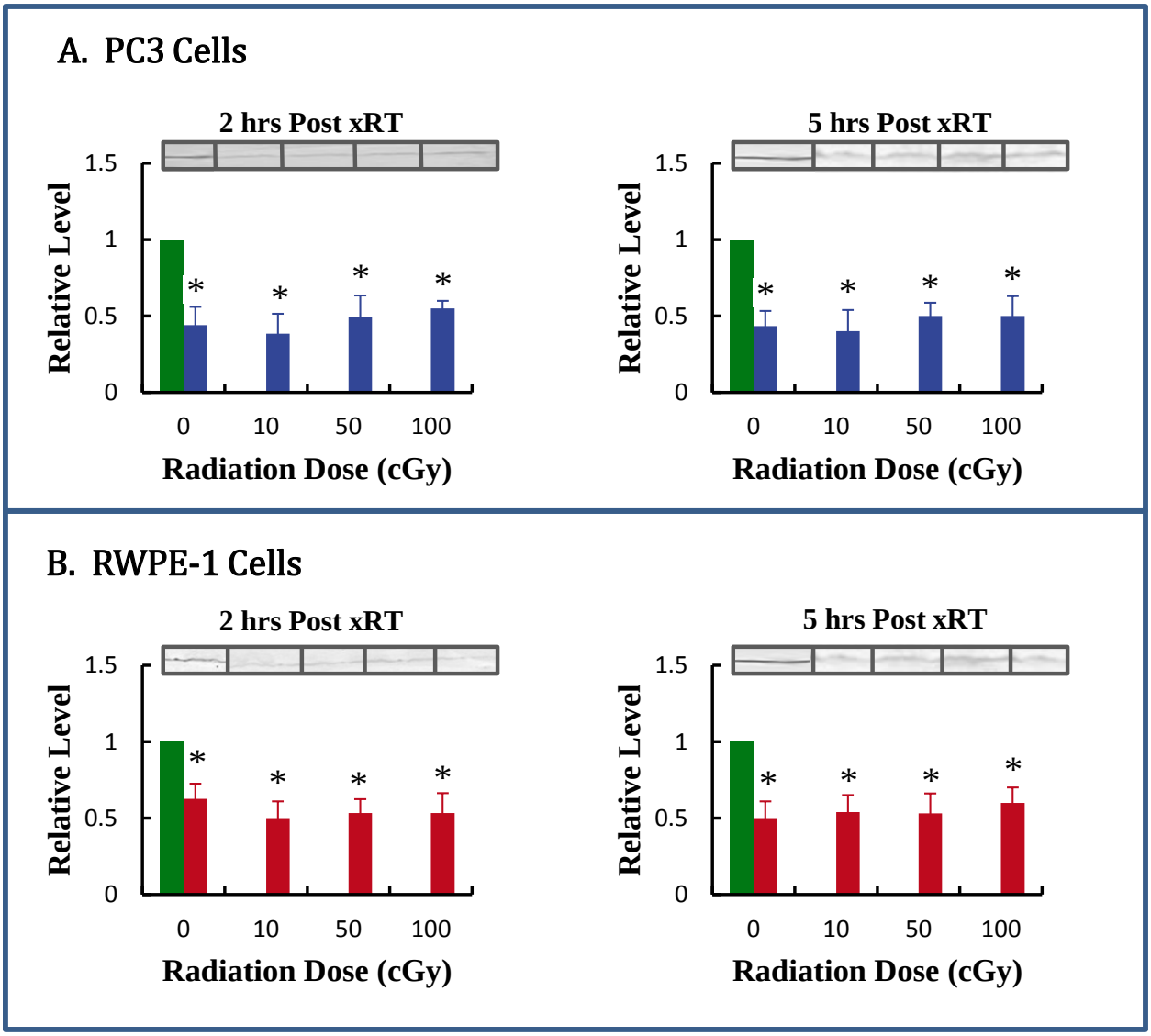


Figure 3.6 Changes in the relative levels of cyclin B1 protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following treatment with xRT + genistein. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. Representative Western blot bands of protein are displayed above each graph. Data demonstrate a significant reduction in cyclin B1 levels (indicated by an asterisk) when compared to untreated control for both PC3 and RWPE-1 cells.

Summary of Relative Cyclin B1 Levels in PC3 and RWPE-1 Cells for All Three Experimental Treatment Groups

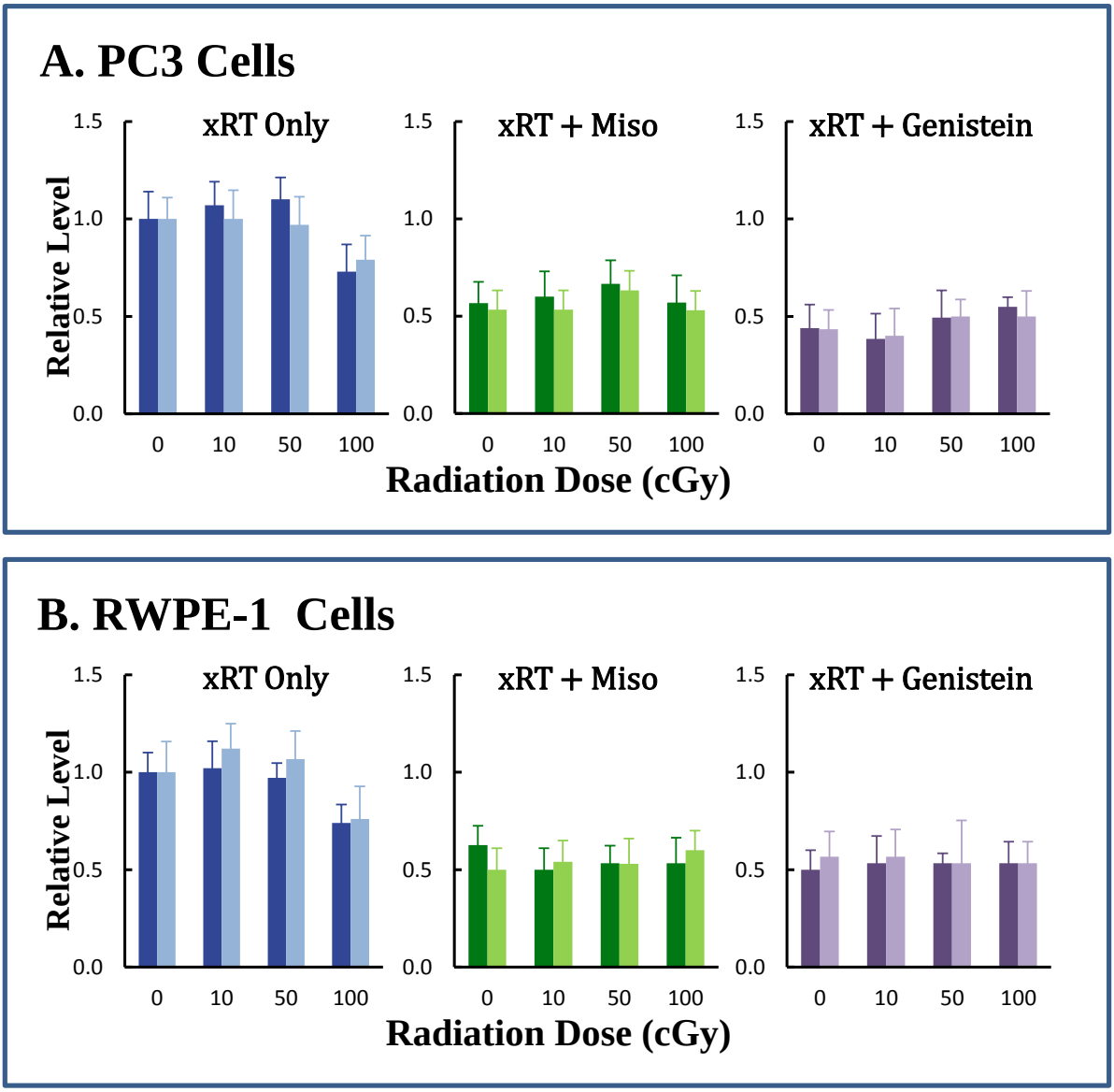


Figure 3.7 Summary of changes in relative cyclin B1 levels in PC3 cells (panel A) and RWPE-1 cells (panel B) for all three treatment groups at both early and late time points. Note that following radiation alone, cell cycle arrest is seen only at 100 cGy, but a significant reduction in cyclin B1 levels following treatments of radiation plus miso and radiation plus genistein occurs at all doses, suggesting the induction of a strong G_2/M block by these compounds that is independent of the radiation-induced block.

3.2.2. Changes in Cyclin A Levels for PC3 and RWPE-1 Cells

To monitor S phase cell cycle blocks that may be occurring as a result of low dose radiation exposure, the levels of the protein cyclin A were measured for the xRT Only treatment group using Western blot analysis. These data are displayed in Figure 3.8 for both PC3 (panel A) and RWPE-1 (panel B). Similar to the studies monitoring cyclin B1, these data represent the mean (±SEM) of three independent experiments with the bands of protein from representative Western blots being displayed above the relative band intensities determined by Image J software. As can be observed, while minor fluctuations from the untreated control level of cyclin A are seen at the various radiation doses assayed, no significant reduction in cyclin A is present for either the tumorigenic PC3 or the non-tumorigenic RWPE-1 cell lines, strongly suggesting that no S phase cell cycle block is occurring as a result of irradiating at these low doses.

Furthermore, the results presented in Figures 3.9 and 3.10 which display the changes in the relative levels of cyclin A following treatment with xRT + miso and xRT + genistein, respectively, indicate that S phase cell cycle blocks in either the tumorigenic PC3 or the non-tumorigenic RWPE-1 cell lines are not being induced as a function of adding either miso or genistein to the low dose radiation exposures.

Finally, for ease of comparison, Figure 3.11 summarizes the relative cyclin A low dose radiation responses (0 - 100cGy) in both the tumorigenic PC3 cells (panel A) and the non-tumorigenic RWPE-1 cells (panel B) for all three treatments groups at both time points monitored. As can be observed, results indicate that little difference is seen in cyclin A levels as a function of (a) the dose of radiation given; (b) the time after radiation at which the cyclin is measured (2 and 5 hours post irradiation); (c) the addition of miso or genistein to the radiation

schedule; or (d) the cell line being investigated (PC3 vs. RWPE-1). These data, therefore, suggest that the induction of a block in the S phase of the cell cycle does not appear to be a mechanism underlying the growth inhibition seen following treatment with these compounds (Figure 3.1) nor does it seem to be involved with either the potential radiopotentiating or antioncogenic responses discussed earlier in this chapter (Figures 3.2 and 3.3).

Relative Cyclin A Levels in PC3 and RWPE-1 Cells Following xRT Only

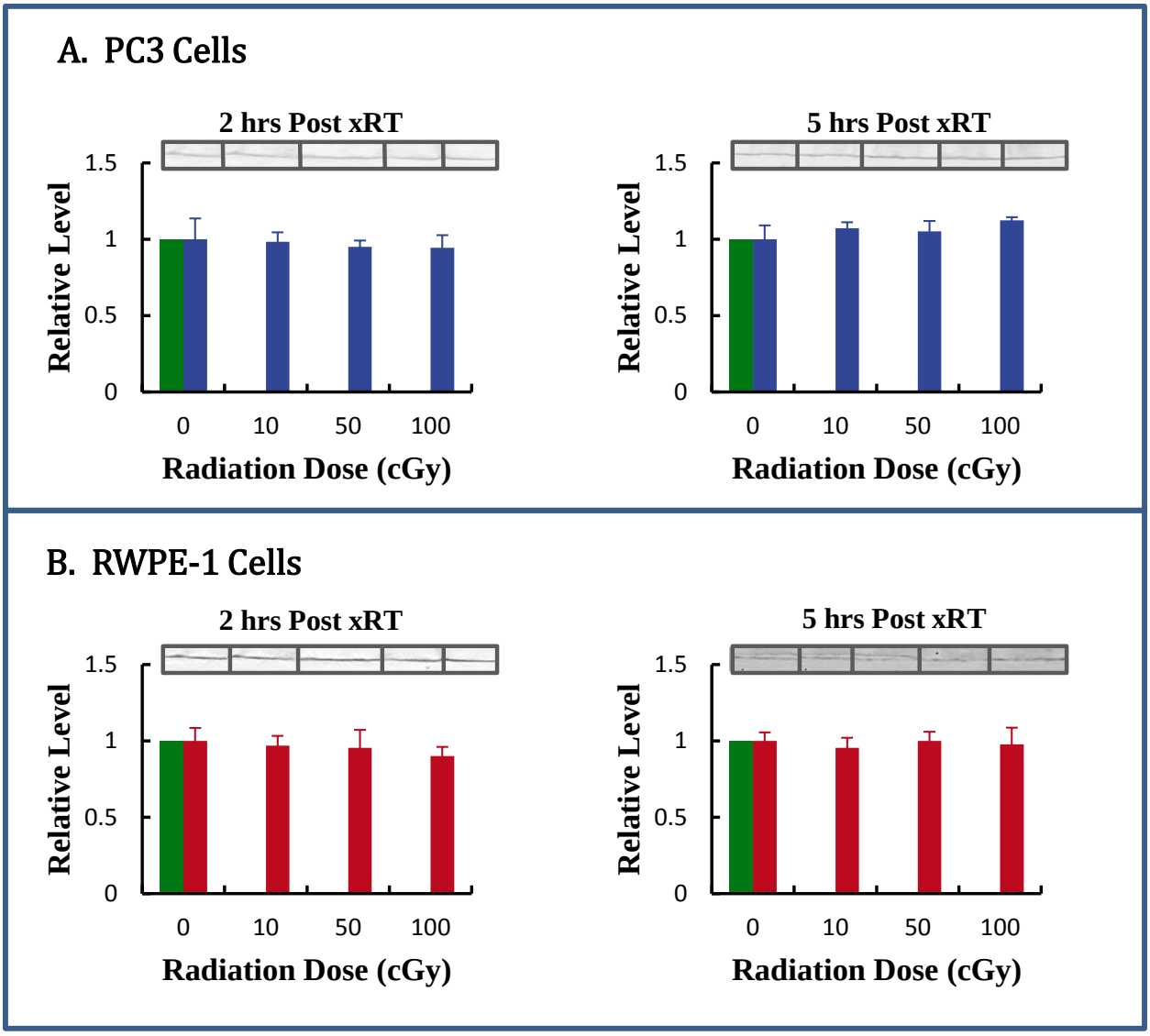


Figure 3.8 Changes in the relative levels of cyclin A protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following irradiation with doses ranging from 0-100 cGy. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. The bands of protein in a representative Western blot are displayed above each graph. The lack of any significant reduction in cyclin A protein in either the PC3 or RWPE-1 cell models suggest that a S phase block is not occurring.

Relative Cyclin A Levels in PC3 and RWPE-1 Cells Following xRT + Miso

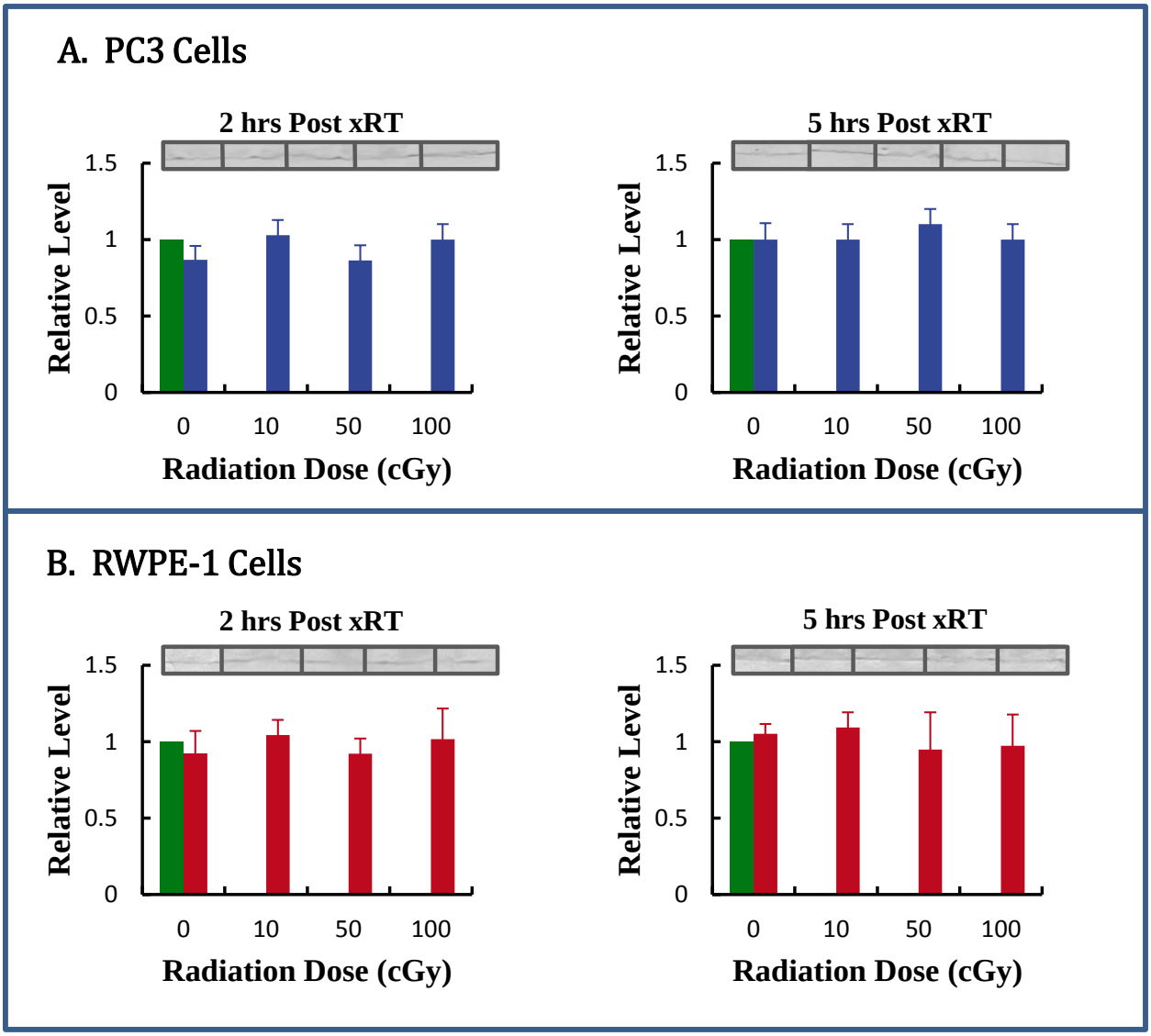


Figure 3.9 Changes in the relative levels of cyclin A protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following xRT + Miso with doses ranging from 0-100 cGy. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. The bands of protein in a representative Western blot are displayed above each graph. The lack of any significant reduction in cyclin A protein in either the PC3 or RWPE-1 cell models suggest that a S phase block is not occurring.

Relative Cyclin A Levels in PC3 and RWPE-1 Cells Following xRT + Genistein

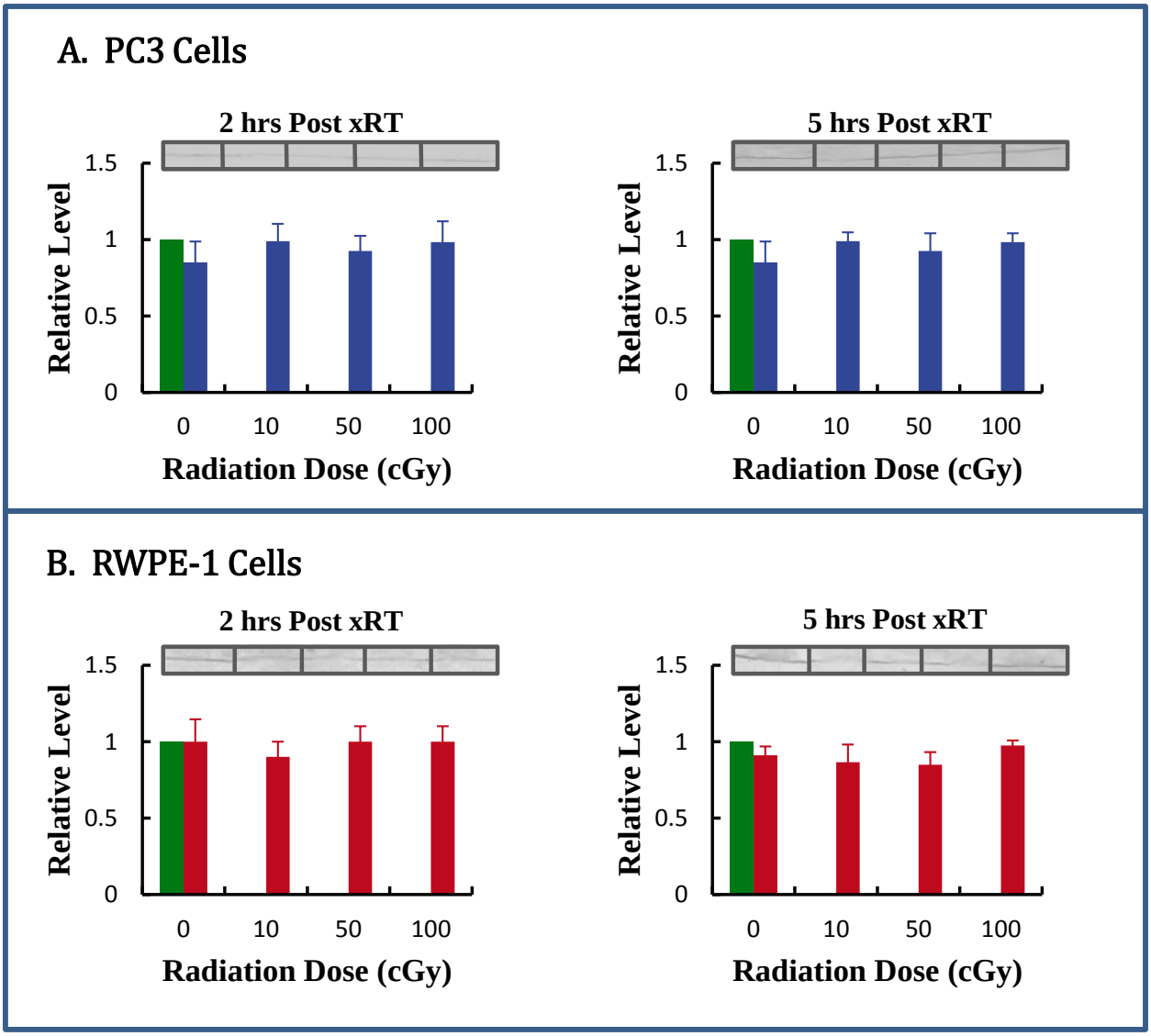


Figure 3.10 Changes in the relative levels of cyclin A protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following xRT + Genistein with doses ranging from 0-100 cGy. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. The bands of protein in a representative Western blot are displayed above each graph. The lack of any significant reduction in cyclin A protein in either the PC3 or RWPE-1 cell models suggest that a S phase block is not occurring.

Summary of Relative Cyclin A Levels in PC3 and RWPE-1 Cells for All Three Experimental Treatment Groups

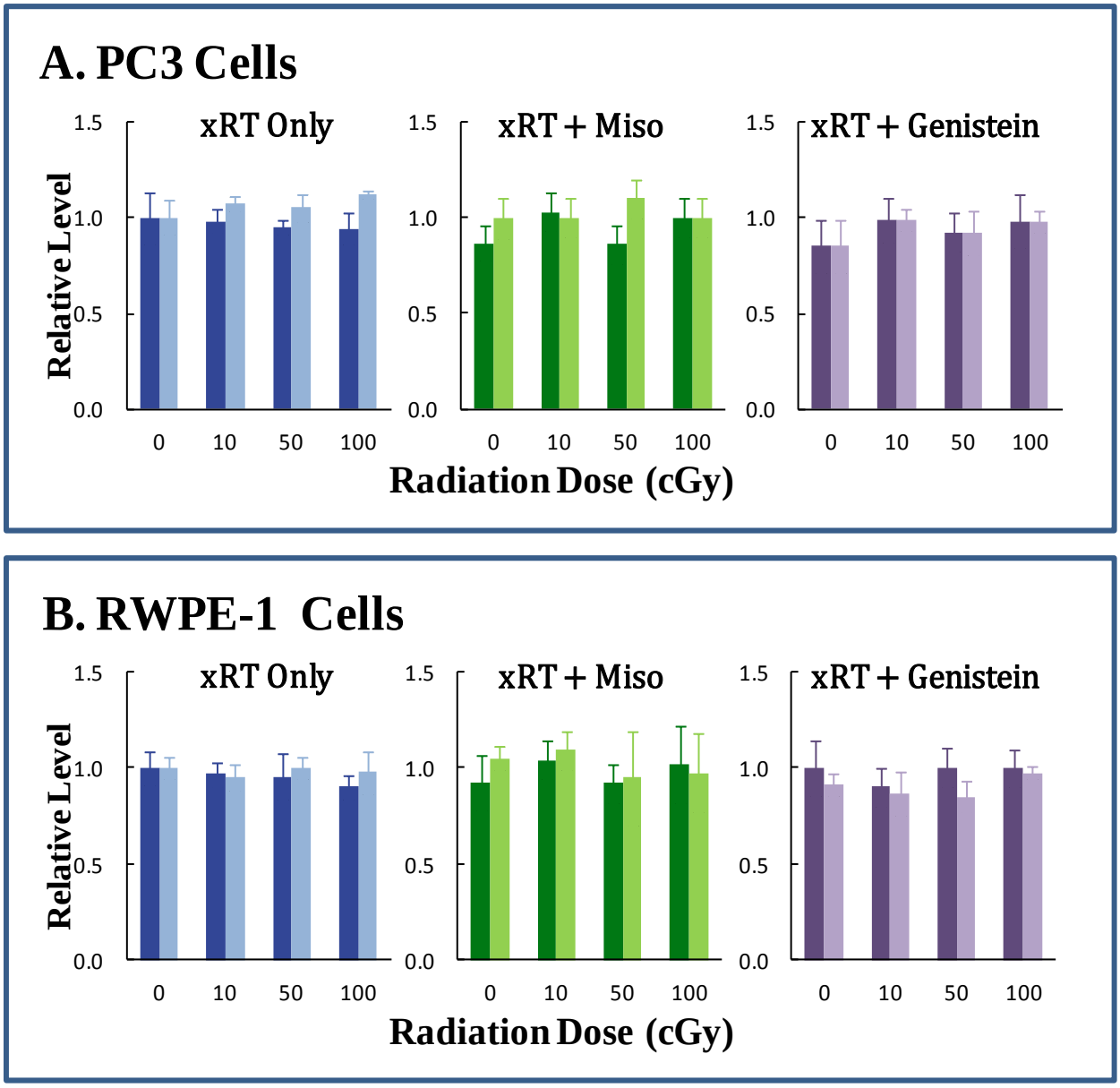


Figure 3.11 Summary of changes in relative cyclin A levels in PC3 cells (panel A) and RWPE-1 cells (panel B) for all three treatment groups at both early and late time points. Note that no significant reduction in cyclin A levels were observed following any treatment protocol, suggesting that a S phase cell cycle block is not occurring.

3.2.3. Changes in Cyclin D1 Levels for PC3 and RWPE-1 Cells

To monitor G_0/G_1 phase cell cycle blocks that may be occurring as a result of low dose radiation exposure, the levels of the protein cyclin D1 were measured for the xRT Only treatment group using Western blot analysis. These data are displayed in Figure 3.12 for both PC3 (panel A) and RWPE-1 (panel B). Similar to the studies monitoring cyclin B1 and cyclin A, these data represent the mean (±SEM) of three independent experiments with the bands of protein from representative Western blots being displayed above the relative band intensities determined by Image J software. As can be observed, the pattern of response was very similar to that seen following analysis of cyclin A responses (Figure 3.8 and 3.11) in that no significant radiation-induced reduction in cyclin D1 was apparent in either the tumorigenic PC3 or the non-tumorigenic RWPE-1 cell lines, indicating that the low dose radiation exposures used in these experiments did not induce a block in the G_0/G_1 phase of cell cycle. Similarly, the results presented in Figures 3.13 and 3.14 which display the changes in the relative levels of cyclin D1 following treatment with xRT + miso and xRT + genistein, respectively, also indicate that G_0/G_1 phase cell cycle blocks are not being induced by these treatment protocols.

A summary of the relative cyclin D1 responses in both the tumorigenic PC3 cells (panel A) and the non-tumorigenic RWPE-1 cells (panel B) for all three treatments groups at both time points monitored is presented in Figure 3.15. As can be observed, results indicate that little difference is seen in relative cyclin D1 levels as a function of (a) the dose of radiation given; (b) the time after radiation at which the cyclin is measured; (c) the addition of miso or genistein to the radiation schedule; or (d) the cell line being investigated (PC3 vs. RWPE-1), indicating that a block in the G_0/G_1 phase of the cell cycle is probably not a mechanism underlying the growth inhibitory, radiopotentiating or antioncogenic responses displayed in Figures 3.1, 3.2 and 3.3.

Relative Cyclin D1 Levels in PC3 and RWPE-1 Cells Following xRT Only

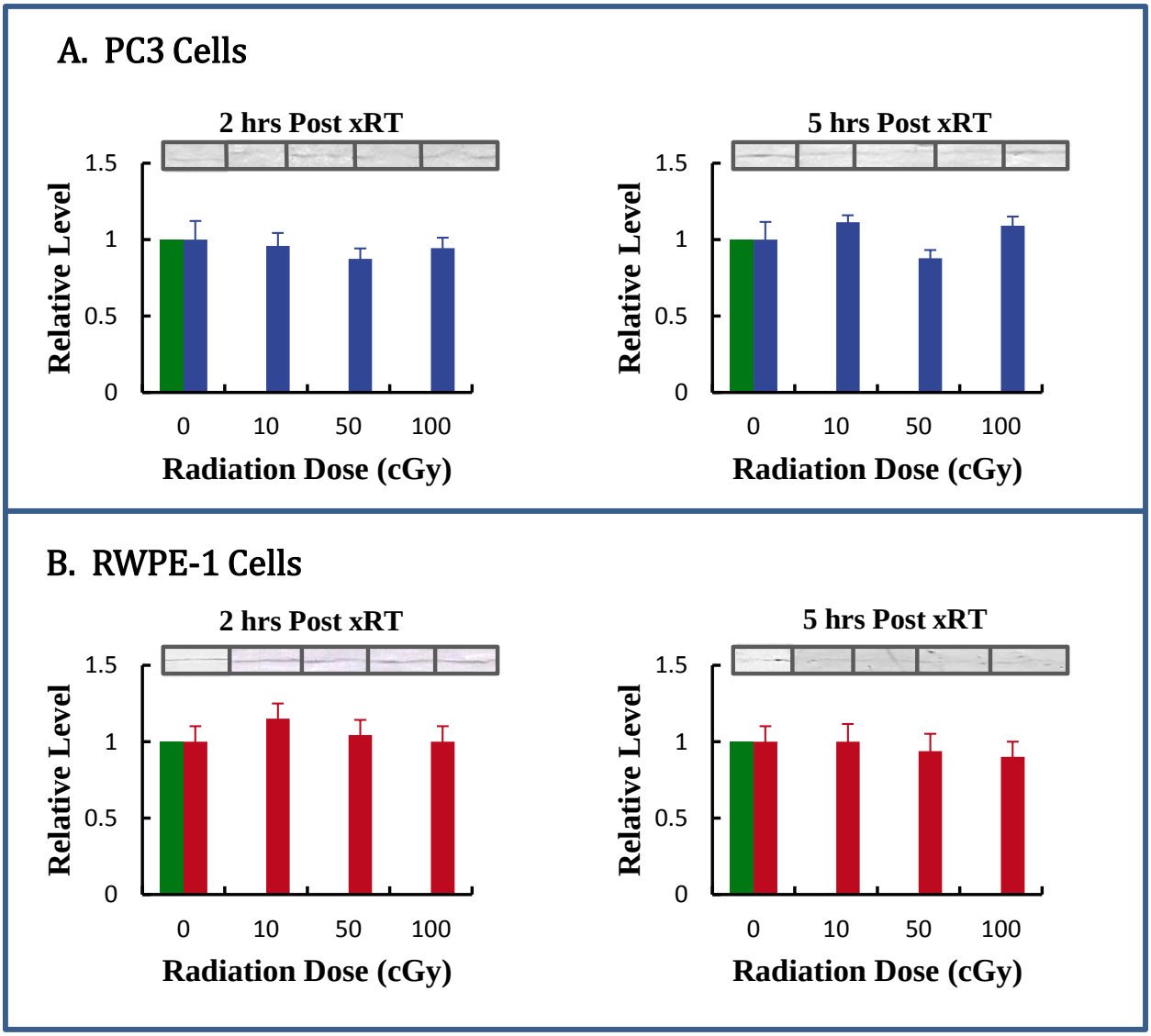


Figure 3.12 Changes in the relative levels of cyclin D1 protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following irradiation with doses ranging from 0-100 cGy. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. Representative Western blot bands of protein are displayed above each graph. Data demonstrate no significant cyclin D1 reduction is observed in either the PC3 or RWPE-1 cells.

Relative Cyclin D1 Levels in PC3 and RWPE-1 Cells Following xRT + Miso

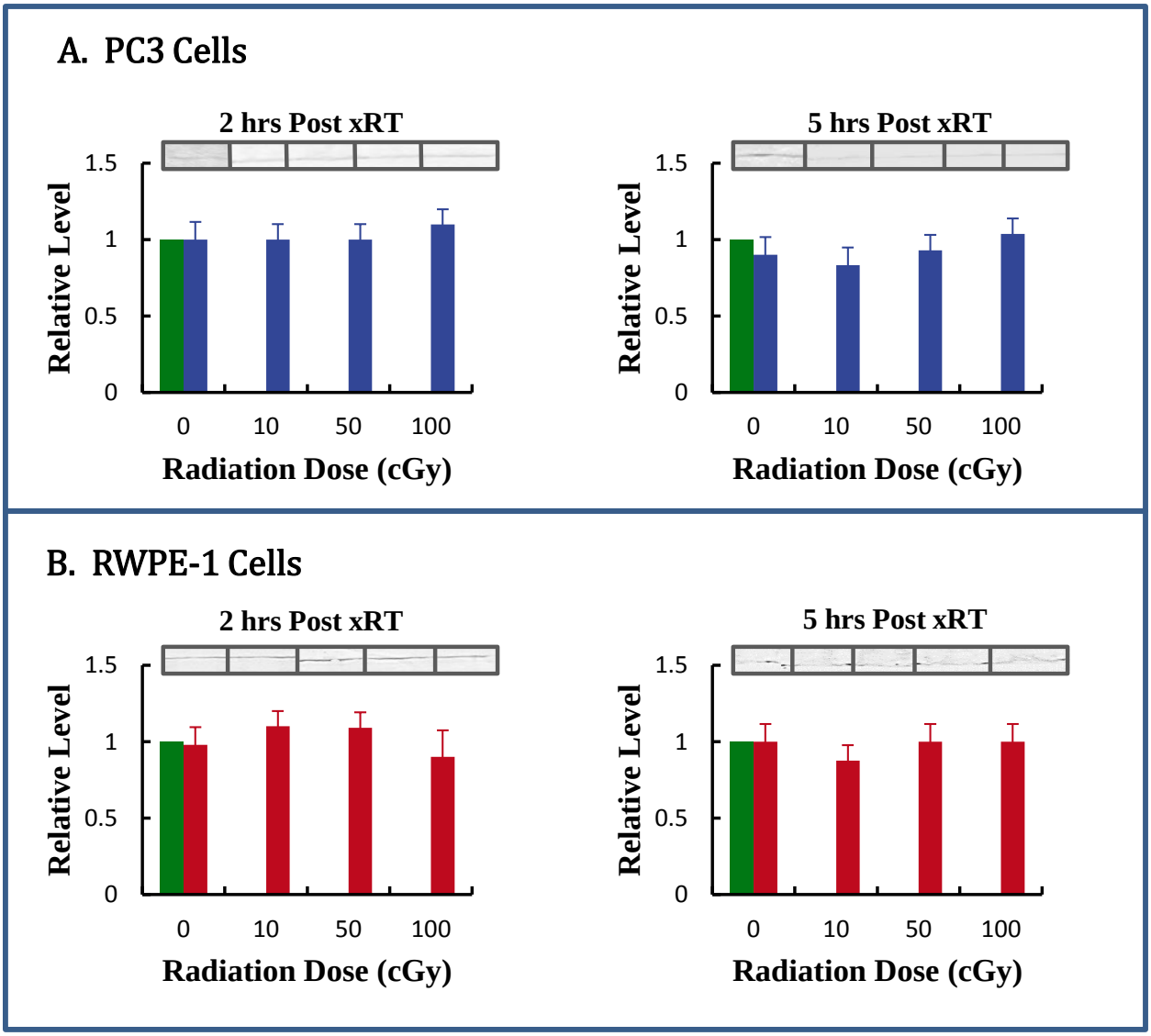


Figure 3.13 Changes in the relative levels of cyclin D1 protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following xRT + Miso treatment. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. Representative Western blot bands of protein are displayed above each graph. Data demonstrate no significant cyclin D1 reduction is observed in either the PC3 or RWPE-1 cells.

Relative Cyclin D1 Levels in PC3 and RWPE-1 Cells Following xRT + Genistein

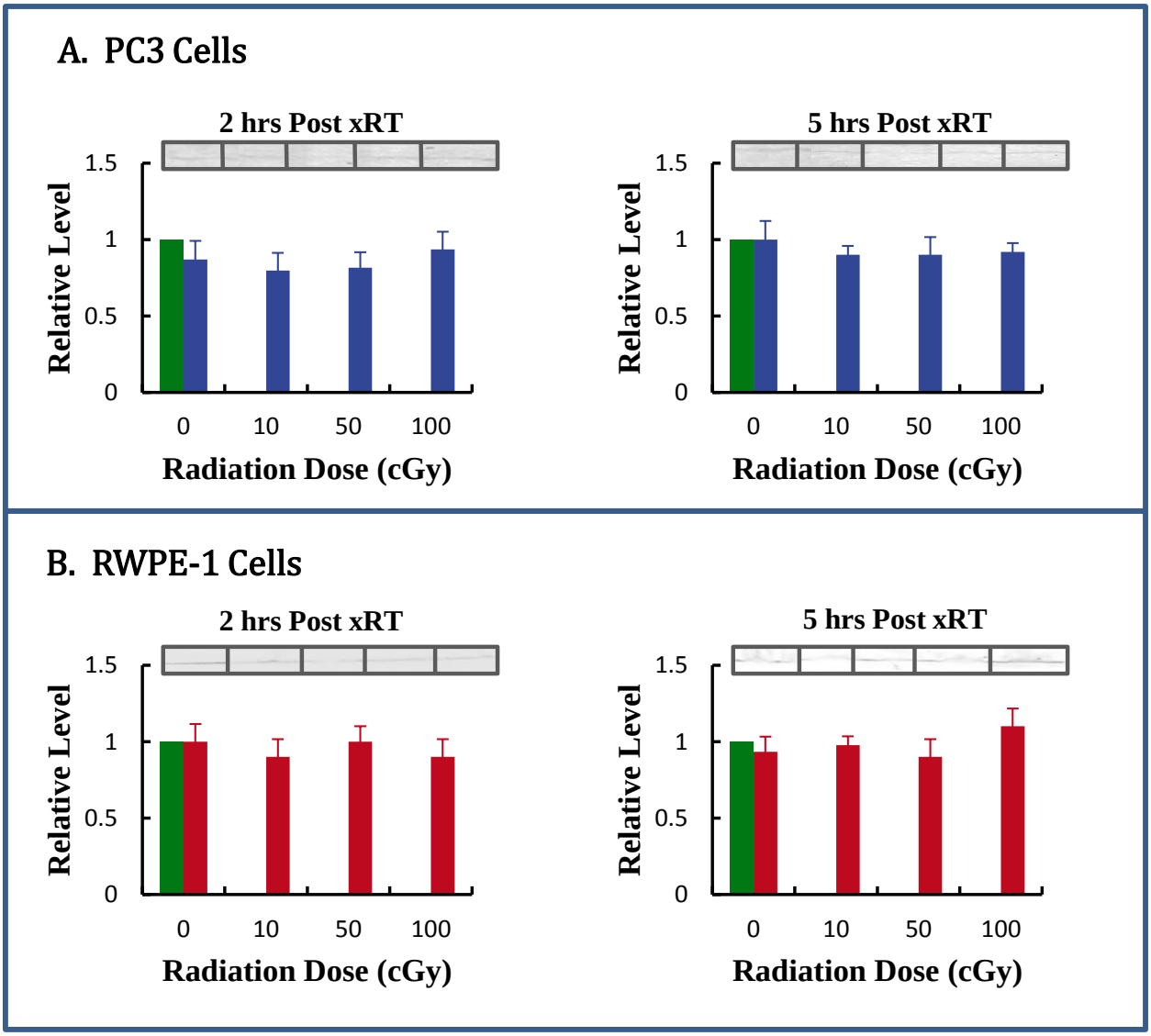


Figure 3.14 Changes in the relative levels of cyclin D1 protein for PC3 (panel A) and RWPE-1 (panel B) cells two and five hours following xRT + genistein treatment. Western blot analysis was performed using Image J software with the graphs representing band intensity (relative to untreated control, green bar). At least three independent experiments (with three different samples for each data point) were performed with the means shown and the error bars representing the SEM. Representative Western blot bands of protein are displayed above each graph. Data demonstrate no significant cyclin D1 reduction is observed in either the PC3 or RWPE-1 cells.

Summary of Relative Cyclin D1 Levels in PC3 and RWPE-1 Cells for All Three Experimental Treatment Groups

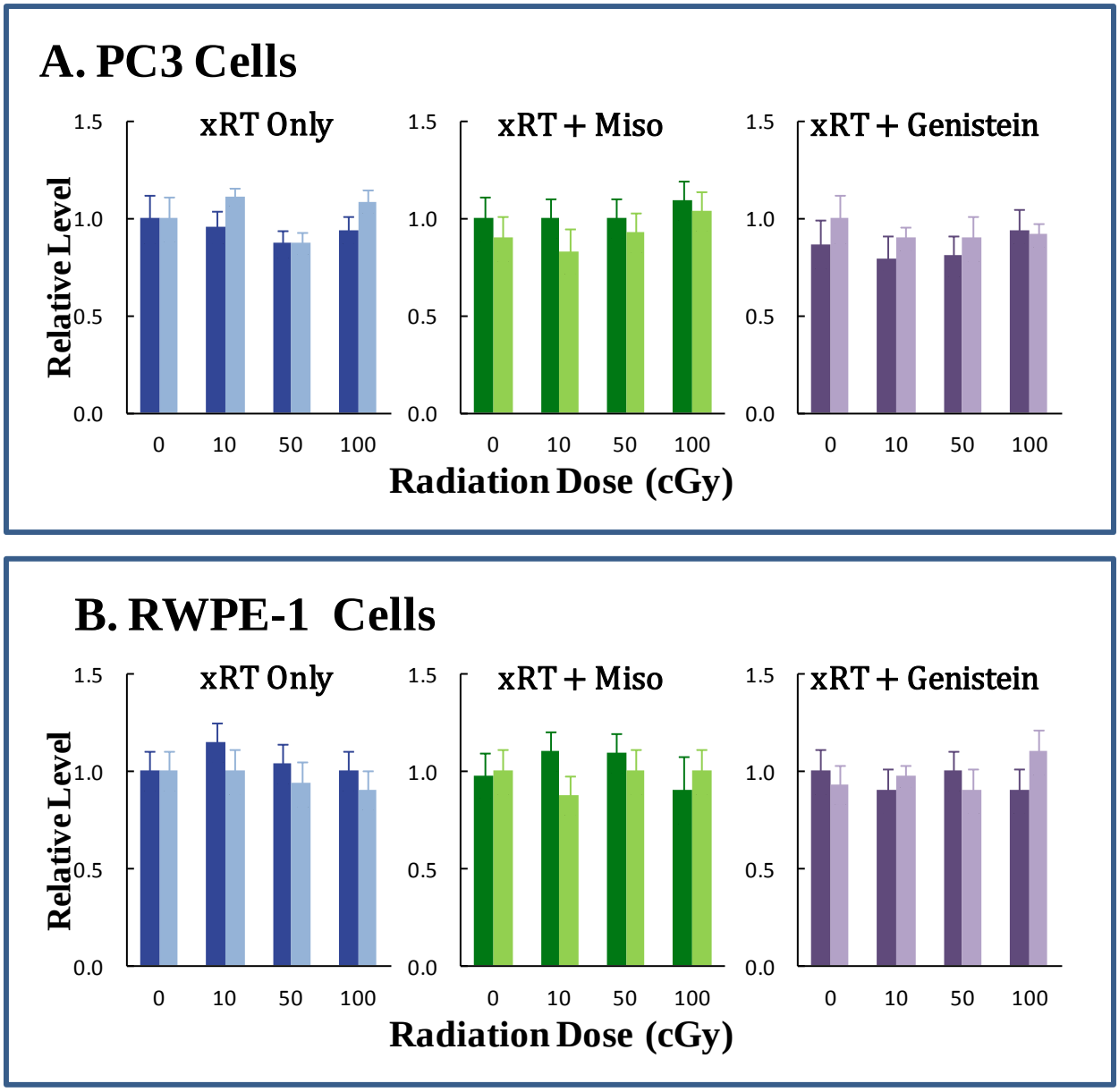


Figure 3.15 Summary of changes in relative cyclin D1 levels in PC3 cells (panel A) and RWPE-1 cells (panel B) for all three treatment groups at both early and late time points. Note that no significant reduction in cyclin D1 levels were observed following any treatment protocol, suggesting that a G_0/G_1 phase cell cycle block is not occurring.

3.3 COMPARISON OF APOPTOTIC PATHWAYS IN PC3 IN RWPE-1 CELLS EXPOSED TO RADIATION AND TREATMENT WITH MISO OR GENISTEIN

Because both radiation and genistein exposures have been reported to elevate the rate of apoptosis [95, 100, 101, 104-106, 108], increased cell death due to apoptosis could be a factor contributing to the growth inhibitory, radiopotentiating and/or antioncogenic effects observed in these studies following administration of miso and genistein prior to low dose irradiation. Therefore, to assess whether the treatment protocols investigated in this study were increasing the rate of apoptosis, elevated signaling through both the extrinsic and intrinsic apoptotic pathways was monitored.

3.3.1 Signaling through the Extrinsic Apoptotic Pathway

To determine whether the rate of apoptosis was increased through elevated signaling via the extrinsic apoptotic pathway, both the latent (pro-) and the activated forms of caspase 8 were monitored. Briefly, caspase 8 is an initiator caspase whose activation early on in the extrinsic apoptotic cascade is often used as a marker of increased signaling through this pathway. Western blot analysis of changes in the concentration of activated caspase 8 for both the tumorigenic PC3 cells and the non-tumorigenic RWPE-1 cell are presented in Figures 3.16 and 3.17, respectively. All blots also include both a negative control (NC), demonstrating the latent pro-caspase 8 band and a positive control (PC), showing both the latent pro-caspase 8 band and the activated caspase 8 bands that are indicative of apoptosis. For the PC3 cells (Figure 3.16), it can be seen that a pro-caspase band is present at both 2 hours post xRT and 5 hours post xRT for all treatment groups at all radiation doses. However, no activated caspase 8 bands are seen for any of the treatment protocols at either time point measured, suggesting that apoptotic signaling through the extrinsic pathway is not occurring. Results for the RWPE-1 cells (Figure 3.17) are essentially identical to the response seen for the PC3 cells, demonstrating no activation of

caspase 8 at either 2 or 5 hours post irradiation for any treatment protocol. These data, suggest that the growth inhibitory, radiopotentiating and antioncogenic effects observed following combination xRT + miso or xRT + genistein are not occurring because of increased signaling through the extrinsic apoptotic pathway.

Caspase 8 Activity in PC3 Cells Following All Three Treatment Protocols

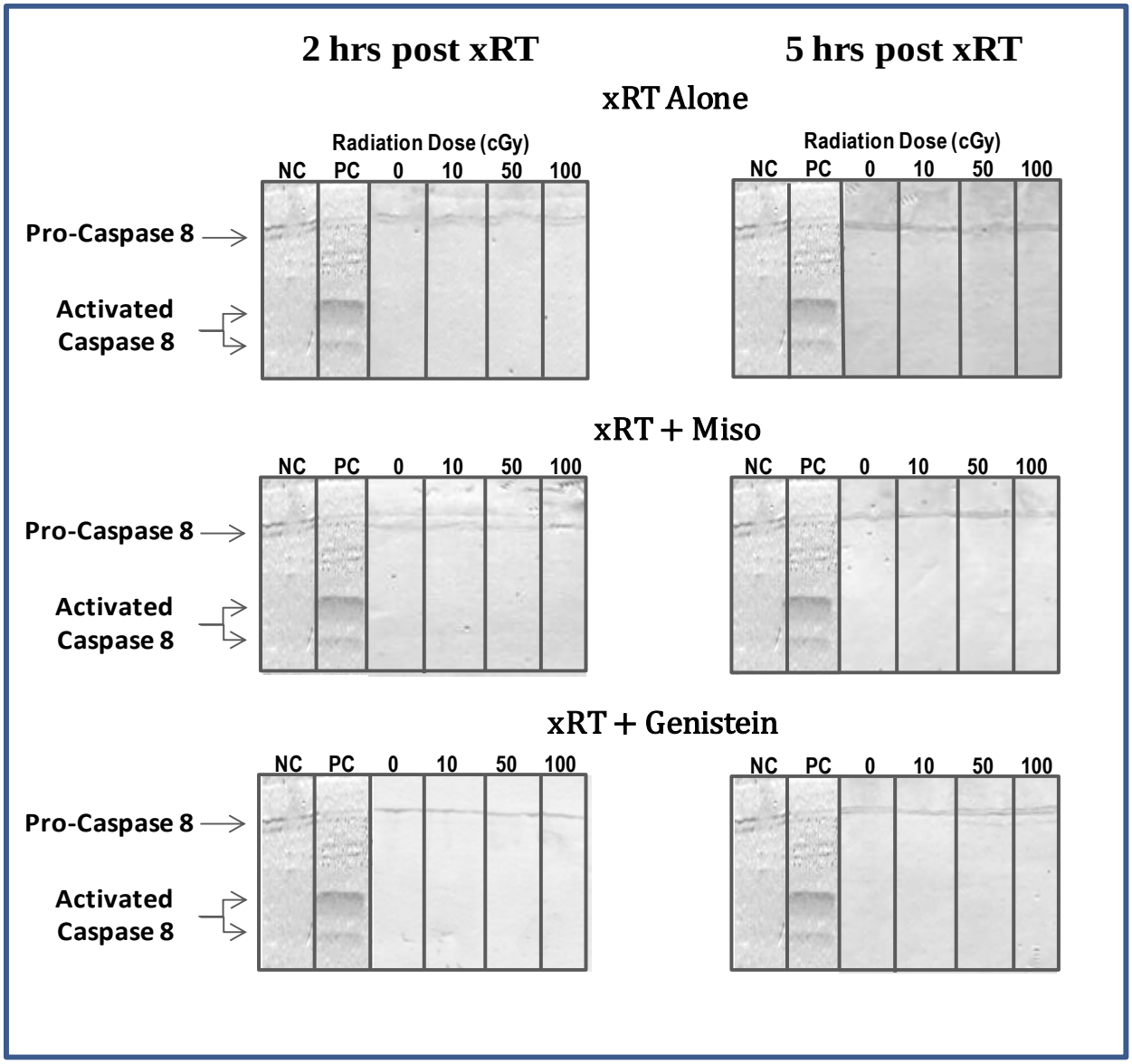


Figure 3.16 Western blot analysis of the extrinsic pathway in PC3 cells treated with xRT alone, xRT + miso, and xRT + 30μM genistein at both 2 and 5 hours post-irradiation. Three independent experiments (±SEM) were performed, and representative Western blots are shown. The column labeled NC is the negative control showing the latent pro-caspase 8 band, while the column labeled PC is the positive control showing cleavage of pro-caspase 8 into two smaller molecular weight moieties that are the activated forms of caspase 8. The radiation doses are labeled above the columns. Data demonstrate that caspase 8 does not appear to be activated following any of the treatment protocols, suggesting that signaling through the extrinsic apoptotic pathway is not occurring.

Caspase 8 Activity in RWPE-1 Cells Following All Three Treatment Protocols

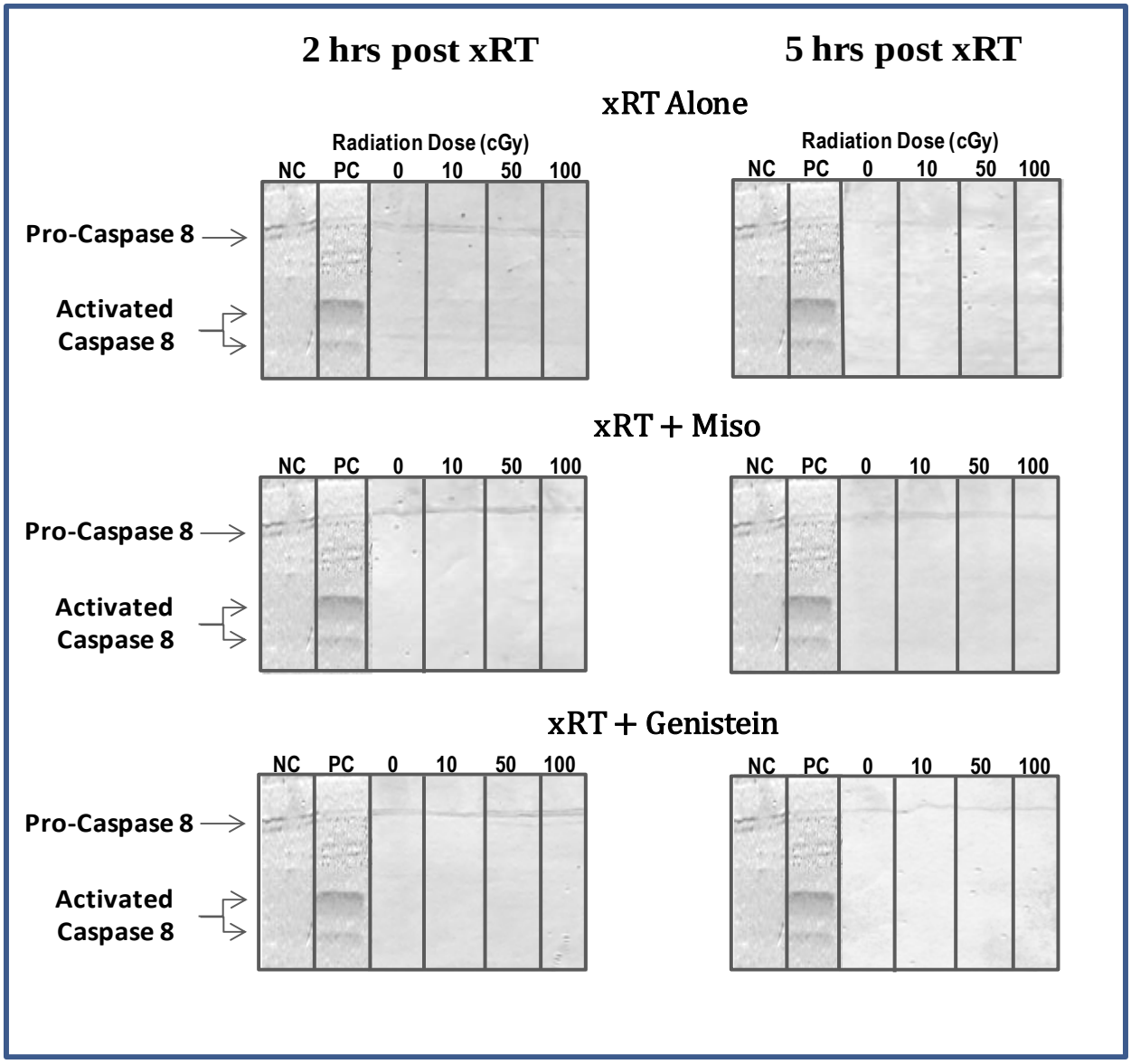
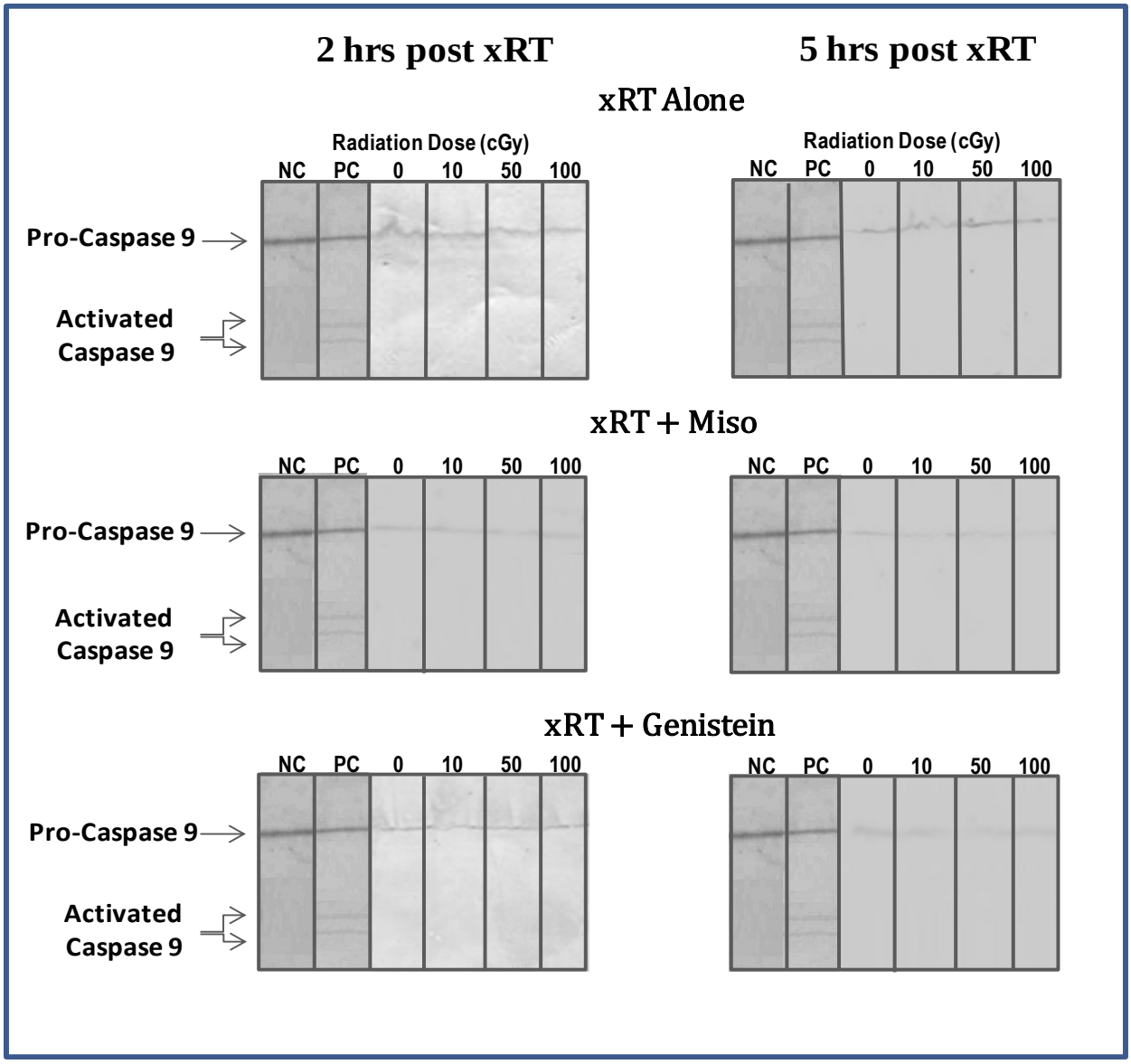


Figure 3.17 Western blot analysis of the extrinsic pathway in RWPE-1 cells treated with xRT alone, xRT + miso, and xRT + 30μM genistein at both 2 and 5 hours post-irradiation. Three independent experiments (±SEM) were performed, and representative Western blots are shown. The column labeled NC is the negative control showing the latent pro-caspase 8 band, while the column labeled PC is the positive control showing cleavage of pro-caspase 8 into two smaller molecular weight moieties that are the activated forms of caspase 8. The radiation doses are labeled above the columns. Data demonstrate that caspase 8 does not appear to be activated following any of the treatment protocols, suggesting that signaling through the extrinsic apoptotic pathway is not occurring.

3.3.1 Signaling through the Intrinsic Apoptotic Pathway

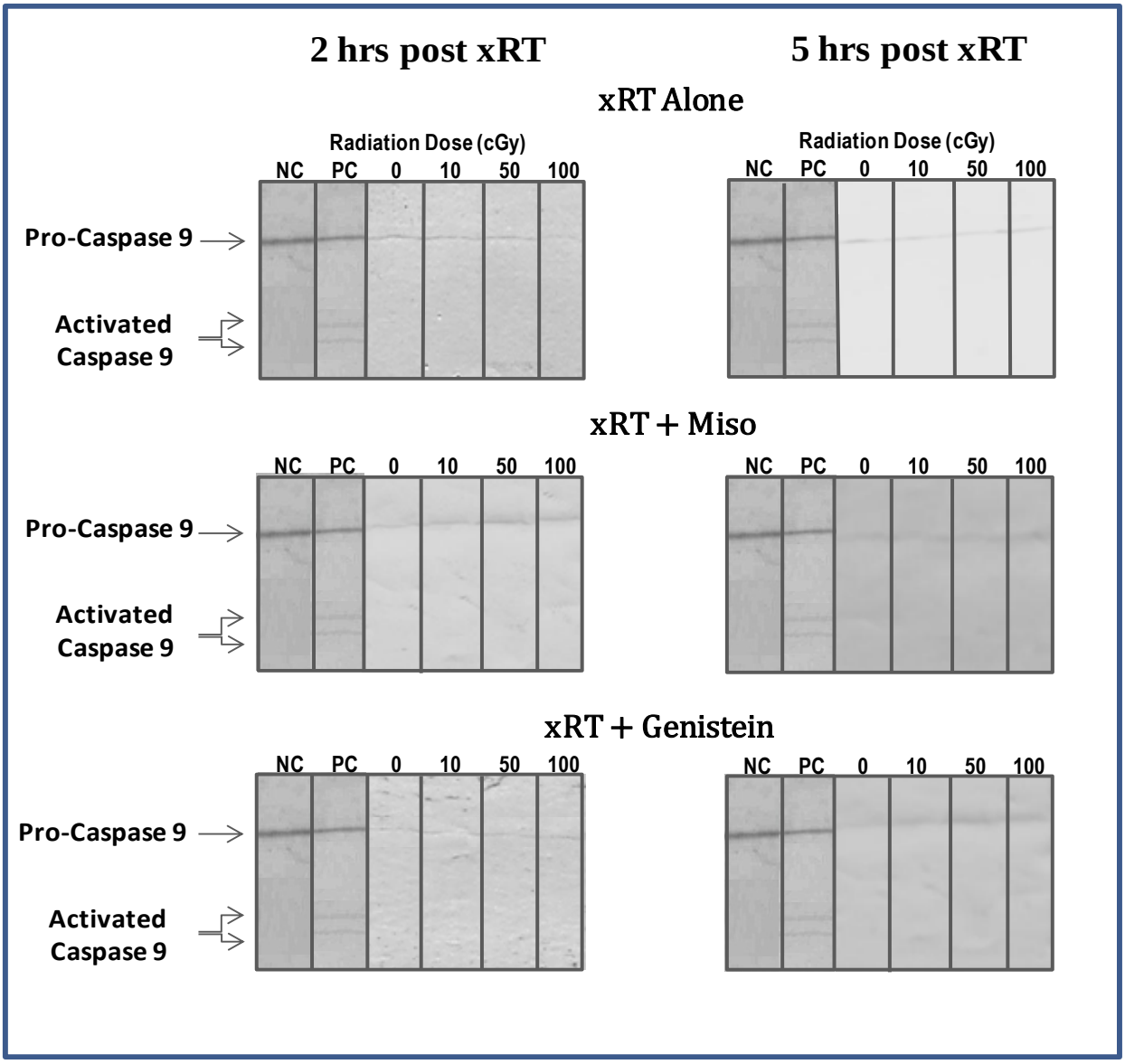
Despite lack of signaling through the extrinsic apoptotic pathway, increased cell death due to apoptosis could still be contributing to the growth inhibitory, radiopotentiating and antioncogenic effects presented in Figures 3.1, 3.2 and 3.3 by increased signaling through the intrinsic apoptotic pathway. Therefore, experiments were also performed to assess whether the treatment protocols were increasing the rate of apoptosis via the intrinsic apoptotic pathway. To accomplish this, both the latent (pro-) and the activated forms of caspase 9 were monitored (*this initiator caspase is a well-known marker of the intrinsic apoptotic pathway*). Western blot analysis of changes in the concentration of activated caspase 9 for both the tumorigenic PC3 cells and the non-tumorigenic RWPE-1 cells are presented in Figures 3.18 and 3.19, respectively. Once again, all blots also include both a negative control (NC), demonstrating the latent pro-caspase 9 band only, and a positive control (PC), showing both the latent pro-caspase 9 band and the activated caspase 9 bands that indicate apoptosis is occurring. As can be seen, and similar to the caspase 8 experiments, results of these caspase 9 studies demonstrated that neither the PC3 cells (Figure 3.18) nor the RWPE-1 cells (Figure 3.19) exhibit activated caspase 9 bands following any of treatment protocols, suggesting that no apoptosis is occurring from elevated signaling through the intrinsic apoptotic pathway. Therefore, increased apoptosis following the radiation plus miso or radiation plus genistein treatments does not seem to be a mechanism underlying miso's and genistein's growth inhibitory, radiopotentiating or antioncogenic effects.

Caspase 9 Activity in PC3 Cells Following All Three Treatment Protocols



3.18 Western blot analysis of the intrinsic pathway in PC3 cells treated with xRT alone, xRT + miso, and xRT + 30μM genistein at both 2 and 5 hours post-irradiation. Three independent experiments (±SEM) were performed, and representative Western blots are shown. The column labeled NC is the negative control showing the latent pro-caspase 9 band, while the column labeled PC is the positive control showing cleavage of pro-caspase 9 into two smaller molecular weight moieties that are the activated forms of caspase 9. The radiation doses are labeled above the columns. Data demonstrate that caspase 9 does not appear to be activated following any of the treatment protocols, suggesting that increased signaling through the intrinsic apoptotic pathway is not occurring.

Caspase 9 Activity in RWPE-1 Cells Following All Three Treatment Protocols



3.19 Western blot analysis of the intrinsic pathway in RWPE-1 cells treated with xRT alone, xRT + miso, and xRT + 30μM genistein at both 2 and 5 hours post-irradiation. Three independent experiments (±SEM) were performed, and representative Western blots are shown. The column labeled NC is the negative control showing the latent pro-caspase 9 band, while the column labeled PC is the positive control showing cleavage of pro-caspase 9 into two smaller molecular weight moieties that are the activated forms of caspase 9. The radiation doses are labeled above the columns. Data demonstrate that caspase 9 does not appear to be activated following any of the treatment protocols, suggesting that increased signaling through the intrinsic apoptotic pathway is not occurring.

CHAPTER 4: DISCUSSION

4.1 LOW DOSE RADIATION CELL SURVIVAL RESPONSE IN PC3 AND RWPE-1 CELLS FOLLOWING TREATMENT WITH MISO OR GENISTEIN

Collectively, the results from the experiments designed to meet the goals of Specific Aim 1 demonstrated the following: 1) no measurable radioprotection was observed in either the PC3 or the RWPE-1 cells treated with genistein or miso prior to irradiation in the low dose radiation range of 0 to100 cGy; 2) on the contrary, a pronounced growth inhibitory effect was observed in both cell lines following treatment with either high dose genistein (30μM) or high dose miso (1:10,000) which was apparent over the entire range of radiation doses administered; 3) a more-than-additive (synergistic) reduction in survival was observed following the combination of radiation with high dose miso or high dose genistein in both experimental cell lines; this synergistic response was found to be much more pronounced in the tumor cell (PC3) model than in the non-tumor cell (RWPE-1) model; and, finally, 4) when comparing the clonogenic survival responses of the experimental treatment groups as function of tumor vs. non-tumor cell types, a strong antioncogenic response was observed.

Lack of Radioprotective Ability – It was the hypothesis of this dissertation that fermented soy miso would be a good radioprotector. As stated earlier, characteristics of an effective radioprotector are that the compound possesses strong radioprotective ability and is also available to the lay public, relatively non-toxic and easy to administer. This need is not met by amifostine, the current "gold standard" in radioprotectors, due to its limited availability and its problems with cytotoxicity at the doses necessary for radioprotection. However, as a common food, miso is readily available, known to be safe and is easily administered. Furthermore, since the first written record in 700 BC, cultures which have used miso as a food, have believed that it possesses remarkable health-promoting properties, including radioprotective properties [83]. For

example, Dr. Shinichiro Akizuki credits miso as the agent protecting the hospital staff, including himself, from the deleterious effects of radiation exposure following the bombing in Nagasaki [83].

However, anecdotal accounts notwithstanding, no well-designed scientific studies were found by this author characterizing the radioprotective abilities of miso. As such, one of the goals of this investigation was to more rigorously characterize miso's radioprotective potential. Unfortunately, the results of these experiments failed to demonstrate any measurable radioprotective ability for miso or for its primary component genistein, at least in the radiation dose range investigated in these studies (see Figure 3.1). The reasons for this response are not clear, but may involve factors such as the experimental model and/or design employed. For example, while little study on miso's radioprotective properties exist, genistein has been comprehensively studied and reported to be a radioprotector in some cases. However, the studies examining genistein as a radioprotective agent concentrated on *in vivo* models such as CD2F1 mice [90, 124] and Swiss albino mice [125], while the studies reported in this investigation are performed on an *in vitro* experimental model. The differences between *in vivo* and *in vitro* models are extensive, especially, considering *in vitro* models are not able to simulate the complex physiological interactions occurring inside an animal. Additionally, differences in the radiation doses administered may account for the lack of radioprotection observed in this investigation, since the radiation doses studied in the mouse studies cited above were much higher than the 0 – 100 cGy range focused on in these experiments. For example, in CD2F1 mice the radiation doses studied were 8.75 and 9.5 Gy, substantially higher than even the highest radiation dose (1 Gy) used in this study. Finally, since it is well known that it is difficult to effectively correlate drug dosages given *in vivo* with those administered to *in vitro* culture

systems, another difference that may be involved in the radioprotection reported in the animal studies versus the lack of radioprotection observed in this investigation may be the differing doses of genistein used.

Growth Inhibitory Action - A second major observation obtained from the experiments presented in Figure 3.1 is the pronounced growth inhibition seen in both PC3 cells and RWPE-1 cells as a result of treatment with either high dose miso (1:10,000 dilution) or high dose genistein (30μM). This growth inhibition is significant for both the tumor and non-tumor cell models used, is present both in the absence of irradiation and following all radiation doses tested, and, finally, is of similar magnitude for both the miso and genistein treatments. Furthermore, the results are in agreement with several reports from the current literature documenting that treatment with genistein or a genistein cocktail before exposure to radiation induces a strong growth inhibitory effect on several cell types such as mouse embryonic fibroblasts (3T3 cells) [98]; human cervical cancer cells (ME180 and CaSki cells) [120]; breast cancer cell lines MDA-MB-231 and MCF-7 [106, 126]; the lymphoma cell line Nb2-11 [127], and, of particular interest in this investigation, the prostate cancer cell lines PC3, LNCaP, and Du-145 as well as the non-tumorigenic prostate cell lines RWPE-1 and CRL-2221 [128-131]. For example, in a study by Raffoul et al., PC3 cells pre-treated with a 30μM genistein cocktail dose prior to a 3 Gy photon radiation had such a pronounced growth inhibition that no clonogenic survival was present [118].

Lack of Stimulatory Action - As stated above, Figure 3.1 demonstrates that the high doses of miso and genistein used in this investigation brought about a growth inhibitory action. The low doses of these compounds did not appear to influence cell survival to any significant extent, either when given alone or in conjunction with radiation. These results were somewhat

surprising in lieu of reports documenting the ability of low doses of genistein to induce a growth stimulatory action. Specifically, growth stimulation has been observed at genistein doses ranging from 1μM - 10μM in a breast carcinoma cell line (MCF-7 cells) and in a ductal breast epithelial tumor cell line (T47D cells) [110, 111]. Additionally, the growth stimulatory effect of low dose genistein has been reported in non-tumorigenic prostate epithelial RWPE-1 cells, where treatment with a 10μM dose of genistein caused a 32% increase in cell proliferation [130]. It is unclear why the results of this investigation do not agree with the above cited studies. Cell specific differences may explain the different responses between the breast cell studies and these data, but would not explain the differential results seen using the prostate epithelial RWPE-1 cell model in this study versus the previously reported RWPE-1 study. A possible explanation in this case may be that the previously reported RWPE-1 study measured cell proliferation, rather than clonogenic cell survival, which was used in this investigation. Specifically, the clonogenic assay, which essentially tests for a cell's reproductive integrity (ability to form a colony over time), has long been accepted as the "gold standard" for determining actual cell survival following irradiation. On the other hand, despite its relative ease of use, the more rapid cell proliferation assays may provide an incomplete assessment of cell killing. It has been argued that, since they assess only events that have occurred up to the time of the assay, they potentially overestimate the amount of cell survival actually present [132]. This overestimation may explain, at least in part, the higher levels of survival seen in the previously reported RWPE-1 study as compared to this study.

Synergistic Action of Combining Radiation and Miso/Genistein - The third major finding from these studies is that observation that a more-than-additive or ***synergistic*** reduction in cell survival appears to result from combining radiation with either high dose miso or high dose

genistein (see Figure 3.2). Once again, little-to-no previous documentation exists regarding miso's action, but these results do support previous reports regarding genistein's radiomodulating action. For example, genistein has been shown to enhance cell killing when combined with radiation in prostate cell models such as the PC3 and DU-145 cell lines [94, 95, 118, 133] with the magnitude of the enhancement increasing as a function of increasing radiation dose. Additionally, genistein's potentiation of radiation effects has been documented for other tissue types as well, including human epithelial cervical cancer cell lines (CaSki and ME180) [120], human non-small-cell lung cancer cell lines, A549 and H1650 [134]; and various esophageal cancer cell lines [135]. Of particular interest, while previous studies have reported synergistic interaction, the studies have concentrated on monitoring radiation doses much higher than those studied in these experiments. This is the first reported instance that miso/genistein potentiates radiation-induced damage at doses of 1 Gy and below.

A second important observation regarding the synergistic action of combined miso/genistein and radiation is that, while the synergism is present in both the tumor and non-tumor cell models, it is significantly more pronounced the tumor cell (PC3) model than in the non-tumor cell (RWPE-1) model. This differential response between tumor and non-tumor models agrees with a previous report in which Li *et al* observed that genistein was a radiosensitizer to PC3 cells, but did not affect the radioresponse of non-tumorigenic CRL-2221 cells [115]. Interestingly, miso and genistein's ability to synergize with radiation in tumor cells to a greater extent than in non-tumor cells may suggest a potential role for these compounds in the radiation oncology clinic as a way to improve the therapeutic index of radiotherapy regimens.

Antioncogenic Action - The fourth major observation derived from this set of experiments became apparent when comparing the clonogenic survival responses of the high dose miso and genistein experimental treatment groups as function of tumor versus non-tumor cell types (see Figure 3.3). Specifically, when comparing the responses of the tumorigenic PC3 cells with the non-tumorigenic RWPE-1 cells following radiation alone, no apparent differences in their clonogenic survival curves is apparent, suggesting that they have virtually identical radiosensitivity profiles. However, when either miso or genistein is added to the radiation regimen, significant cell type specific differences are readily apparent, with the non-tumor RWPE-1 model demonstrating much less cell kill than is seen in the tumor PC3 model. These results indicate that combination miso/genistein and radiation regimens may have a strong antioncogenic potential which could potentially be used to increase the vulnerability of tumor tissues with similar detriment to normal tissues. Although this is the first report of antioncogenic action of miso found in the literature, similar reports have been found for genistein. For example, although not involving radiation, Li and Sarkar's study comparing the response of tumorigenic PC3 cells with non-tumorigenic CRL-2221 cells to treatment with genistein documented a pronounced antioncogenic effect for this compound [115]. In fact, the promising antioncogenic abilities of genistein have led to clinical trials to ascertain the efficacy of this compound in humans. Currently, the NCI is sponsoring phase I, II, and III clinical trials, studying the effects of soy isoflavones, including genistein, on the chemoprevention of breast cancer [136]. In addition, phase II clinical trials, also sponsored by the NCI, are underway to study the effects of genistein and other soy isoflavones on the prevention of prostate cancer [137].

4.2 CELL CYCLE CHANGES IN PC3 AND RWPE-1 CELLS AFTER LOW DOSE RADIATION AND TREATMENT WITH MISO OR GENISTEIN

One aim of this dissertation was to investigate the radiomodulating effects of miso and genistein treatment on the expression of cell cycle regulator proteins in an effort to correlate the growth inhibitory, synergistic and antioncogenic effects of these compounds with the downregulation of cyclin levels. As stated previously, the rationale for choosing to monitor cyclin protein levels is based on the crucial role that the cyclin /CDK complexes play in triggering the progress of cells through the various phases of the cell cycle. Specifically, the reduction of cyclin levels within the cell decreases the amount of cyclin/CDK levels available and, as a result, triggers cell cycle arrest.

Collectively, the results from these studies demonstrated the following: 1) neither exposure to radiation nor administration of genistein or miso induced reductions in the relative levels of cyclins A or D1, suggesting that no significant evidence of G_0/G_1 or S phase arrest is seen in either PC3 or RWPE-1 cells as a result of these treatments; 2) however, for both cell lines, radiation-induced reductions in cyclin B1 levels were observed at 100 cGy, indicating that a G2/M block is occurring, although the lack of a block at 50 cGy and below suggests that it is dose dependent; and 3) in both the tumorigenic and non-tumorigenic cell models, both the radiation + miso and radiation + genistein experimental groups demonstrated a marked reduction in cyclin B1 levels over the whole spectrum of radiation doses given, suggesting that a G_2/M phase cell cycle block is occurring as a function of the miso or genistein administration which is independent of the radiation-induced arrest.

As stated previously, numerous studies have shown that radiation stress interferes with the normal progression of the cell cycle in a variety of cell lines [138-140]. Indeed, cell cycle arrest after exposure to radiation is the norm, rather than the exception, with several studies

documenting the presence of arrests in all three phases of the cell cycle following irradiation, although G2/M is the most commonly reported block [141-144]. For example, for HeLa cells reports have documented blocks in S phase after irradiation with 500 cGy [145, 146], blocks in G_2/M phase after irradiation with doses between 34 -135 cGy [147], and blocks in G_0/G_1, S and G_2/M phases following irradiation with 300 cGy [148, 149]. Similarly, studies using Chinese hamster cells exposed to a series of doses between 1.5-6 Gy demonstrated dose dependent division delay in all three cell cycle phases [150]. It is, therefore, not surprising that a strong, dose dependent G2/M block was observed in these two cell lines following radiation stress, but the lack of evidence for a G_0/G_1 or S phase arrest is less well understood. Because most reports on radiation-induced blocks in G_0/G_1 or S phase have been on exposures greater than 100 cGy, it may be that the low doses used in this study simply were not sufficient to induce a block in these phases.

Another major finding of this set of studies was that in both the tumorigenic PC3 and the non-tumorigenic RWPE-1 cell models, both the radiation + miso and radiation + genistein experimental groups demonstrated a marked reduction in cyclin B1 levels. Although no reports are available documenting miso's effects on the cell cycle, the genistein data from these studies are in agreement with previous studies on PC3 cells in which a G_2/M block was observed following treatment with genistein [95, 151, 152]. Additionally, several other cell lines have reported that genistein induces a cell cycle arrest, including a human gastric cancer cell line (HGC-27) [123]; a T-cell leukemia cell line (Jurkat) [153]; and the breast cancer cell lines T47D and MDA-MB-231) [154, 155]. Of particular interest in these studies is the observation that the cyclin B1 reductions after administration of genistein or miso occur following treatment with these compounds alone as well as over the whole spectrum of radiation doses given. These

results suggest that this block may be, at least in part, responsible for both the reduced cell survival seen in the radiation + miso and radiation + genistein experimental groups (see Figure 3.1) and the potential synergistic growth inhibition observed when either of these treatments is combined with radiation (see Figure 3.2). However, the lack of significant differences in the magnitude of cyclin B1 reduction between the PC3 and the RWPE-1 cell lines following either radiation + miso or radiation + genistein suggests that the antioncogenic action of these compounds is most likely occurring through some other mechanism.

4.3 COMPARISON OF APOPTOTIC PATHWAYS IN PC3 AND RWPE-1 EXPOSED TO LOW DOSE RADIATION AND TREATMENT WITH MISO OR GENISTEIN

Both radiation and genistein exposures have been reported to elevate the rate of apoptosis [95, 100, 101, 104-106, 108] in several cell lines. In an effort to correlate the growth inhibitory, synergistic and/or antioncogenic effects of these compounds, therefore, with increases in the level of apoptosis, the final aim of this dissertation was to investigate the radiomodulating effects of miso and genistein treatment on the expression of mediators of both the intrinsic and extrinsic pathways of apoptotic signaling. The results of this set of studies demonstrated that caspase 8 (see Figures 3.16 and 3.17), an indicator of apoptotic signaling through the extrinsic pathway, and caspase 9 (see Figures 3.18 and 3.19), an indicator of apoptotic signaling through the intrinsic pathway, were not activated by any of the experimental treatments investigated, indicating that apoptosis did not appear to be an underlying mechanism for the clonogenic survival responses observed in Figures 3.1, 3.2, 3.3. Induction of apoptosis by miso has not been previously studied, but the lack of evidence for increased apoptotic activity in these studies following either radiation exposure or genistein administration was surprising, since it disagrees with several reports in the literature which document a strong apoptotic action for two agents.

For example, genistein has been reported to induce apoptosis in a wide variety of different cancer cell lines including breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and MDA-MB-46 [156]; a T-cell leukemia cell line [153], and a colon cancer cell line [157]. Of particular interest to this study, it was also reported to induce apoptosis on prostate cancer cell lines, specifically the PC3 and RWPE-1 cells, used in these studies. In the PC3 cell study, however, a dose of 50μM genistein was used, and, in the RWPE-1 study, a dose of 100μM genistein was used, so the differential response seen in these studies as opposed to the studies reported above could be due to the much smaller dose of 30 μM used in this dissertation. Additionally, increased apoptosis in PC3 cells treated with 30μM genistein and radiation has been reported previously, but the radiation dose used was 3 Gy, a much higher dose of radiation that the dose range used in this investigation (0 – 100 cGy), and one which is known to induce much more apoptosis [95]. Additional studies using miso and genistein at higher doses would be of interest to elucidate the role increased apoptotic signaling may play in the radiomodulating activity of these two compounds.

In conclusion, the results of these studies suggest that, while meeting many of the criteria for an ideal radioprotector, no radioprotection was observed by either miso or genistein. However, experiments with both miso and genistein demonstrated a strong growth inhibitory action which was independent of radiation. Experiments also demonstrated a synergistic cytotoxic response (i.e. growth inhibition) when miso or genistein were combined with radiation, and experiments also demonstrated the antioncogenic action of both miso and genistein as evidenced by a more pronounced effect on tumor cells than on non-tumor cells. Overall, these findings, therefore, not only point to a way to improve the efficacy of radiotherapy regimens for prostate cancer through the use of miso and genistein's radiopotentiating actions, but also

suggest these compound's antioncogenic properties could potentially be exploited as a novel approach to the prevention of prostate cancer through regular consumption of miso as a dietary supplement.

4.4 FUTURE DIRECTIONS

Based on the results of this investigation, a series of studies to investigate the radiomodulating properties of genistein in the treatment of prostate cancer stem cells have been designed. To date, most studies on prostate cancer have been performed using bulk cancer cells or cell lines. Few studies have investigated genistein's effects on the cancer stem cells themselves, although recent evidence suggests that an understanding of this tumor cell component is critical to gaining insight into the problems of radioresistant residual disease and local recurrence. After enriching for the PC3 cancer stem cell sub-population, initial experiments plan to determine if genistein, when given in conjunction with radiation, will induce growth inhibition and potentiate radiation-induced cytotoxicity in a similar manner as it has for the bulk PC3 cell population used in this study. If successful, subsequent studies will focus on preclinical investigations which center on therapeutic targeting of prostate cancer stem cells and, ultimately, on clinical trials.

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