Multiple dietary supplement and protection against radiation-induced damage

By

Fatemeh Soleimani

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science (MSc) in Biology

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	Multiple dietary supplement and protection against radiation-induced damage
Name of Candidate Nom du candidat	Soleimani, Fatemeh
Degree Diplôme	Master of Science
Department/Program Département/Programme	Biology
Date of Defence Date de la soutenance	September 22, 2016

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. TC Tai
(Supervisor/Directeur(trice) de thèse)

Dr Doug Boreham
(Co-supervisor/Co-directeur(trice) de thèse)

Dr. Mazen Saleh
(Committee member/Membre du comité)

Dr. Christopher Phenix
(External Examiner/Examinateur externe)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarères
Dean, Faculty of Graduate Studies
Doyen, Faculté des études supérieures

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

Abstract

Radiotherapy is a critical part of cancer treatment. With the recent medical advancements and increased survival rate of cancer patients, there is also an increased risk of radiation-related tissue damage and toxicity which could lead to severe organ damage or even organ failure. Medications that could be used as prophylaxis or treatment provide a better quality of life for cancer patients. In the present study, we evaluated the radio-protective potential of multiple dietary supplement (MDS) in an animal model by looking at the gene expression levels of the renin-angiotensin system. In mice receiving 5 Gy radiation, MDS administration as prophylaxis or treatment was able to decrease the expression levels of angiotensinogen which suggested the lower activity of RAS in irradiated kidney tissue. This finding indicates MDS potential for tissue radioprotection. Study of expression levels of kidney antioxidant enzymes also suggested benefits of MDS in protecting kidney tissue from radiation-induced reactive oxygen species evidenced by the lack of upregulation in expression levels of genes such as GPX1, NOS3 and SOD2 in mice receiving MDS as prophylaxis. Also, systemic effects of MDS to protect the body from radiation-induced physiological stress was studied by evaluating genes involved in catecholamine biosynthesis pathway in adrenal. Data from expression levels of phenylethanolamine N-methyltransferase (PNMT) suggests MDS protected the animal from radiation-induced physiological stress. MDS was useful both for prophylaxis and treatment. Further examination was also conducted to determine MDS effects on radiation-induced antioxidant and DNA damage and repair response and also changes in expression levels of DNA methyltransferases. Collectively our results suggested MDS has the potential to protect the mice tissue from radiation induced tissue damage, oxidative stress and physiological stress.

Acknowledgment

This work would not have been possible without the gracious efforts of many wonderful people. First I would like to express my deepest gratitude to my supervisor Dr. T.C. Tai and my co-supervisor Dr. Douglas Boreham who provided me with trust and support that I needed to fulfil this research. I was privileged to work in a wonderful and professional environment and I am grateful for the guidance I've received every step of the way. This work would have never been accomplished without the chance and opportunity that they've given me. I will be forever grateful.

I would like to thank Dr. Sujeenthar Tharmalingam for his amazing and selfless effort to teach me and guide me from the beginning to the very end. He was a wonderful teacher and a great example of excellence at his work. He couldn't be more generous with his gift of knowledge.

I would like to thank my committee member Dr. Mazen Saleh for providing me with feedbacks and guidance.

I would like to thank everybody at NOSM. Especially wonderful members of Tai lab. They are my great friends and colleagues and will be missed dearly. My special thanks to Dr. Sandhya Khurana for her inspiration and support and to Chad Williamson for his help with tissue lysing protocol.

My special thanks to Sandy Jouet, my friend and colleague for her participation in the initial steps of project.

My special Thanks to Dr. Jen Lemon at McMaster University for collecting and shipping the samples.

I would like to express my gratitude to Dr. Mery Martinez Garcia for her tremendous help and support through my transition and Dr. David Lesbarreres for providing me with financial support during the past year. Your moral and professional support provided me the chance to have a fresh start and fulfil something wonderful.

Finally, I gratefully thank Laurentian University faculty of Graduate studies and Department of Biology and Canadian Institutes of Health Research for the financial support.

Table of Content

Abbreviations

ACE	angiotensin converting enzyme
ACEIs	angiotensin converting enzyme inhibitors
ACTH	adrenocorticotropic hormone
Ang I	angiotensin I
Ang II	angiotensin II
AT1 receptor	angiotensin II type 1 receptor
AT1RA	angiotensin Type 1 Receptor Antagonists
AT2 receptor	angiotensin II type II receptor
ATP	adenosine triphosphate
CDKN1a	cyclin-dependent kinase inhibitor 1A
cDNA	complementary DNA
CRF	corticotropin-releasing factor
Ct	cycle threshold
dATP	deoxyadenosine triphosphate
DBH	dopamine β-hydroxylase
dCTP	deoxycytidine triphosphate
DDC	DOPA decarboxylase
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNMT1	DNA methyltransferase I
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
DNMTs	DNA methyltransferase family
DSBs	double-strand breaks
DTC	coiled convoluted tubule
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EGR1	early growth response 1
ENaC	epithelial sodium channels
eNOS	endothelial Nitric oxide synthesis
EtBr	ethidium bromide
ETC	electron transport chain
GADD45	growth Arrest and DNA Damage
GPX1	glutathione peroxidase 1
GR	glucocorticoid receptor

GR	glucocorticoid receptors
H•	hydrogen free radical
HPA axis	hypothalamic-pituitary-adrenal axis
HRR	homologous recombination repair
HSD11B1	11β hydroxysteroid dehydrogenase
iNOS	inducible nitric oxide synthase
KeV	Kilo electronvolt
MDS	multiple dietary supplement
mRNA	messenger RNA
NADPH	nicotinamide adenosine dinucleotide phosphate
NF-κB	NF-kappaB
NHEJ	nonhomologous end-joining
NO•	nitric oxide
NOS	nitric oxide synthases
NOS3	nitric Oxide Synthase 3
NRF2	nuclear factor like-2
$O2^-$	superoxide
•OH	hydroxyl radicals
ONOO-	peroxynitrite
PCR	polymerase chain reaction
PCT	proximal convoluted tubule
PER2	Period circadian clock 2
PNMT	phenylethanolamine N-methyltransferase
PVN	paraventricular nucleus of hypothalamus
qPCR	quantitative PCR
R2	coefficient of correlation
RAS	renin angiotensin system
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
SOD1	superoxide Dismutase 1
SOD2	superoxide Dismutase 2
Sp1	specificity protein 1
SSBs	single-strand breaks
TCA	tricarboxylic acid
TH	tyrosine hydroxylase
TRI	trizol

List of Tables

List of Figures

1.0 Introduction

1.1 Ionizing Radiation and Its Effects on Biological Systems

In the last century, ionizing radiation has been used for a variety of reasons ranging from weapons of war, fuel for the nuclear reactor to diagnostic radiography and cancer therapy. Our exposure to radiation is not limited to these sources as we are exposed to radiation through the natural background radiation, radiation in workplaces and accidental radiation exposures due to disasters caused by damages to nuclear centers. Exposure to radiation leads to two main classes of effects, deterministic and stochastic. Deterministic effects happen above a threshold and occur due to cell killing effects of radiation which could lead to tissue damage and organ failure. The second main class is the stochastic effects of radiation. These effects are not dose dependent and result from radiation-induced nuclear damage which leads to mutation or cancer (1). Radiation-induced deoxyribonucleic acid (DNA) damage may lead to cancer progression (2). Cancer is one of the major consequences of radiation exposure. Radiation therapy could also lead to secondary cancer in patients. Carcinogenesis is a multistep and multifactor process. Radiation's direct effect can cause mutation in the DNA as its major target. Moreover, radiation also causes excessive production of reactive oxygen species (ROS). If cell's antioxidant system can not neutralize ROS, it causes altered gene expression by affecting genetic and epigenetic pathways (3). If cells are unable to repair the damage from radiation or any other insult, they undergo apoptosis. Cancer results when damaged cells fail to die by apoptosis and contain mutations that could cause increased cell growth rate (oncogenes) or damage pathways which suppress the cell growth (tumor suppressor genes) (4).

1.2 Physical Properties of Ionizing Radiation

Ionizing radiation refers to radiation waves which carry enough energy so after interaction with an atom it can dissociate electrons from the orbit and cause the atom to become charged or ionized. Types of ionizing radiation include alpha particles, beta particles, neutrons, gamma rays and x-rays. Alpha radiation or alpha particle is a mass of two protons and two neutrons and positively charged. Due to its relatively high mass and charge, alpha radiation's kinetic energy is lost rapidly and is readily absorbed by materials. Beta radiation is high energy electron formed by nucleus decay and has more power to penetrate and ionize materials compared to alpha radiation. Gamma and x-ray radiation are very high energy massless photons and are highly penetrating in the human body. X-ray is powered by the acceleration of the electron in an electrical device whereas gamma rays are emitted due to nuclear decay in radioactive isotopes. The last type of ionizing radiation is neutron radiation which is produced by ejection of neutrons from nucleases undergoing fission. Materials like water which is abundant in hydrogen atoms can absorb neutron radiation.

Ionizing radiation carries energy and upon exposure to other materials, energy is absorbed (dose). Absorbed dose is determined by the energy in joule deposited in 1 kg of material. This unit is equivalent to 1 Gray. Radiation affects biological systems differently, depend on radiation and tissue type. To get a better understanding of doses of radiation which convey similar degree of harm in biological systems, equivalent dose is generated by multiplying radiation weighting factor by absorbed dose. Equivalent dose is expressed in a measure called Sievert (Sv) (5).

To show how radiation can pose danger to overall health, considering the difference in tissue sensitivity to radiation, effective dose was developed by multiplying equivalent dose by tissue weighing factor (W_T) (6) .

1.3 Sources of Ionizing Radiation

1.3.1 Natural Sources of Radiation or Background Radiation

Background radiation is the constant and low dose radiation humans receive from the environment on a daily basis. There are four major sources of background radiation that include cosmic radiation, terrestrial radiation, or radiation from inhalation and ingestion of radioactive elements.

Terrestrial radiation consists of a low dose (average 0.5 mSv/y) of ionizing radiation released during the natural decay of elements such as uranium, thorium and potassium. These elements are found abundantly in the earth's crust.

Cosmic radiation is another source of high energy rays which mostly consist of protons originated from sources like the sun and other stars which enter the earth's atmosphere. Ozone absorbs some of this radiation and the rest passes through the atmosphere and could be absorbed by the human body. The amount of cosmic radiation varies greatly by location and altitude. For instance, locations at sea level like British Colombia receive 0.30 mSv/y cosmic radiation whereas this amount almost triples at Yukon with an altitude of 2000 m from sea level.

Inhalation is another source of background radiation (1.2 mSv/y). Radioactive gasses such as radon are produced by the decay of a radioactive uranium which is readily present in bedrocks and soil. Thus, there is a high level of the radioactive gas in uranium mines. The

accumulated gas is hazardous to miners as well as people who reside in such regions due to the accumulation of radon in homes.

Mammals are also naturally exposed to ionizing radiation through food and drinking water (0.3 mSv/y). Soil and water contaminated with radioactive isotopes include potassium-40 and carbon-14 and ultimately affects humans through the food chain.

1.3.2 Artificial Sources of Radiation

There is a variety of usage for radiation. For instance in medical diagnostic imaging, x-ray imaging (chest x-ray 0.1 mSv) and Chest CT scan (7 mSv) are routinely used for diagnosis. Another important application of radiation is as radiotherapy which can be given as monotherapy or with surgery and systemic therapy in cancer treatment. Radiation palliative treatment is given when disease is incurable. Different sources of ionizing radiation used for cancer treatment including x-ray, gamma rays, electron, proton and neutron particles. The duration of treatment can range from a single treatment up to eight weeks of daily irradiation (between 40-60 Gy).

Another modern use of radioactive materials is in industries, nuclear power plants (1 mSv/y) and also their use in weapons all contribute to human radiation exposure.

1.4 Radiation Interaction in Biological Systems

1.4.1 Direct Effects of Radiation

Cellular components absorb radiation energy. Direct effects occur when absorbed energy damages and breaks chemical bonds in DNA, membrane lipids and proteins. DNA is a major target for radiation as radiation biological effects are mainly due to the damage to the DNA

(Figure 1) DNA breaks can be base damage, single-strand breaks (SSBs) or double-strand breaks (DSBs). Number of these breaks depends on the radiation dose delivered. In biological systems, SSBs damages are detected and corrected by using the opposite strand very quickly after the damage occurs and biologically not important in the case of radiation's cell killing effects. DSBs are responsible for the most important biological effects of radiation and consists of breaks in both strands of DNA located at opposite side of the DNA or just a few nucleotides apart. Two basic mechanisms are responsible for DSBs repair in eukaryotes. Type of repair depends majorly on the availability of an undamaged homologous chromatid/chromosome to be used as a template. Homologous recombination repair (HRR) happens when a homologous chromosome is available (when damage happens in S/G_2 phase) and non-homologous end-joining (NHEJ) occurs in the G_1(7).

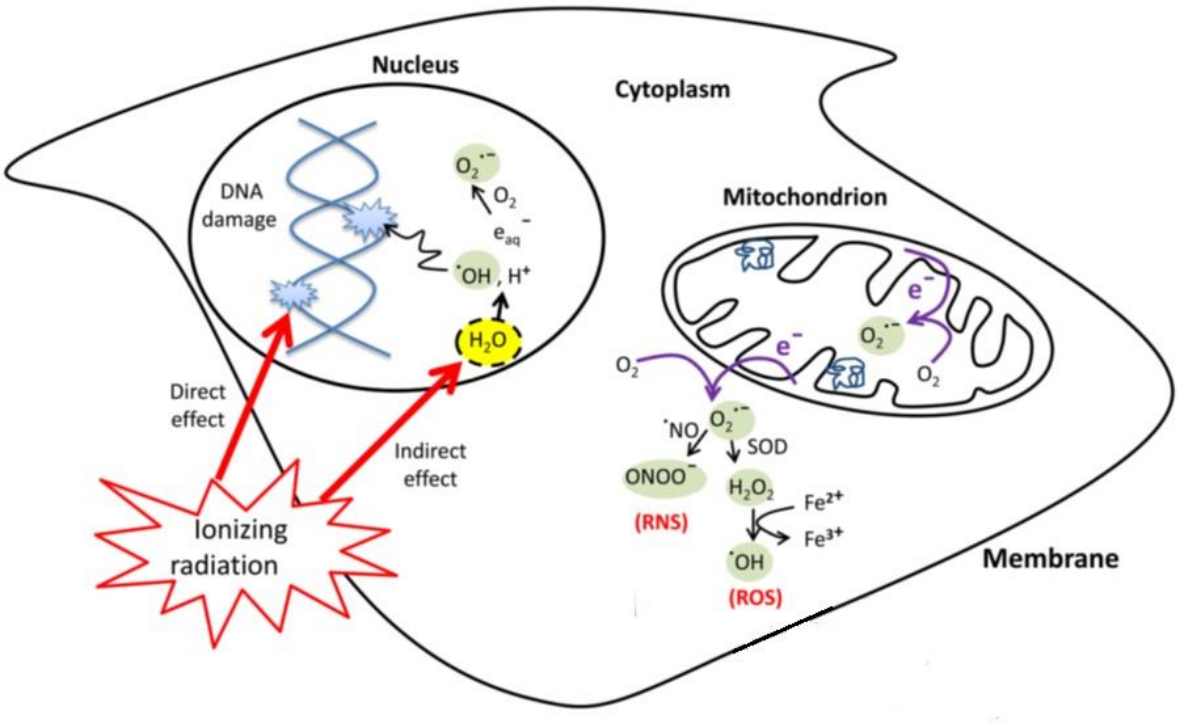

Figure 1 Direct and indirect effects of radiation on cell (16)

1.4.2 Indirect Effects of Radiation

Radiation can also indirectly alter cellular components through ROS. Cells are composed of 80% water. When water absorbs radiation, free ion radicals carrying unpaired electrons are produced (Figure 1). Ion radicals (charged particles) have a very short lifetime (10-10 s) so it decays and leads to the production of free radicals (contains an unpaired electron in the outer shell). Reactive free radicals react with other water molecules resulting in the production of reactive hydroxyl radicals (•OH). This reactive species can diffuse throughout the cell and disrupt the DNA. Almost two third of x-ray damage to DNA is due to hydroxyl radicals (7).

1.5 Radiation-Induced Tissue Damage

To effectively use radiation in treating tumors, inevitably some of the normal tissue surrounding the tumor might be irradiated. Radiation's side effects on the tissue might appear clinically weeks or months after treatment, whereas pathological process leading to it has begun immediately after exposure. To reduce the damage tolerated dose for different organs has been identified and considered as a dose-limiting factor in designing the radiation treatment.

1.5.1 Acute Effects of Radiation

Acute effects of radiation appear shortly after exposure and includes damage to the skin and gut tissue, inflammation and erythema in skin and pneumonia (8, 9).These effects may take days or weeks to start and is observed predominantly in tissues with high cell turnover and cell division. Clinical symptoms appear due to the loss of cells as functional units of the

tissue. In cases like epithelial tissue, the proliferation of stem cells substitute for the lost cells and recovery follows soon after (10).

1.5.2 Radiation-induced late Effects

Late effects of radiation appear months to years after tissue exposure. These symptoms are prominent in tissues with low cell turnover such as cells located in the nervous system, kidney, muscle, etc. Pathology seen in late tissue damage includes fibrosis, necrosis, atrophy and vascular damage. In conclusion, radiation-induced late tissue injury is the manifestation of normal healing process affected by radiation. Disruption of normal healing process results in the failure of tissue to recover and establish its function efficiently (11).

1.5.3 Consequential late effects

As a result of more aggressive radiation treatment regimens, severe cases of acute tissue damage might fail to heal properly and develop to the continuation of early effects which is known as consequential late effects. Skin, mucosal, intestinal and urinary tissue are more prone to show this type of damage. For instance, acute oral mucositis forms in head and neck cancer patients which may result in non-healing ulcers and necrosis (10, 12)

1.6 Inflammation and Radiation-Induced Tissue Damage

Radiation induces production of pro-inflammatory, pre-fibrotic cytokines and vascular injury. These changes are important in radiation-induced acute as well as late effects. Radiation-induced late effects have been proposed to be partly due to activation of pro-inflammatory processes. Radiation-induced inflammation is dose dependent and involves

activation of proinflammatory cytokines. In a normal inflammatory process, the inflammation subsides by activation of anti-inflammatory cytokines. However, radiation-induced inflammation does not mitigate sufficiently by time and could accumulate in the tissue. The overproduction of inflammatory cytokines in the irradiated tissue results in fibrosis (13).

1.7 Radiation-Induced Oxidative Stress and Cellular Damage

Ionizing radiation absorption by cellular molecules causes the production of free radicals, but due to the abundance of water (80%) in cells, water radiolysis generates a vast quantity of reactive oxygen species and free radicals, such as hydrogen free radical (H•), OH•, superoxide ($O2^-$) and H_2O_2 (14).

Radiation also causes the production of vast quantity of nitric oxide (NO•) free radicals by affecting the inducible nitric oxide synthase (iNOS). Although NO• reacts with superoxide anion with a rate constant higher than enzymes such as superoxide dismutase (SOD) which catalyzes the conversion of superoxide to hydrogen peroxide (15, 16). Radiation-induced cellular damage can affect different components and organelles in cells and may be reversible or irreversible. For instance, radiation-induced DNA damage, if not properly repaired leads to cell death within few cell divisions after exposure. In the following section, we discuss few of these radiation damage targets including membrane and mitochondria in more details.

1.7.1 Membrane Disruption

Ionizing radiation can affect cell membrane directly or indirectly through the action of free radicals. Non-lethal doses of ionizing radiation induce reversible changes in the cell membrane. Radiation-induced cell membrane damage is manifested mainly in decreased content and distribution of negatively charged components of glycocalyx including sialic acid, lectin, calcium binding sites, changes in cell surface morphology (rounding up or blebs) and altered cellular communication. Glycocalyx is a carbohydrate coat on the outer surface of the cell membrane which is composed of carbohydrate components (oligosaccharides) of glycolipids (combination of oligosaccharides and lipids covalently bind together) and transmembrane glycoproteins content of cell membrane (17).

1.7.2 Radiation-induced effect on the mitochondria

Mitochondria produces the majority of energy needed for cellular function by metabolizing carbohydrates, fatty acids and amino acids through oxidative phosphorylation of tricarboxylic acid (TCA) cycle in the mitochondrial matrix. Mitochondria is a natural source of ROS. ROS is produced during adenosine triphosphate (ATP) synthesis in the inner mitochondrial membrane or due to the premature leakage of electrons majorly from complex I and III (II to a very limited extent). Radiation exposure further increases the ROS production in mitochondria by increasing the electron leakage from electron transport chain (ETC) and production of superoxide anions. Excessive ROS in mitochondria causes mitochondrial DNA damage or mutation, change in mitochondrial DNA copy number, and alters or damages gene expression and induces nucleus DNA damage (16).

1.8 Local Renal Renin-Angiotensin System and Radiation-Induced Tissue Damage

Although we do not have a clear understanding of the role of renin-angiotensin system (RAS) in radiation-induced tissue damage medications such as angiotensin converting enzyme inhibitors (ACEIs) and angiotensin type 1 receptor antagonists (AT1R antagonists) are being used to treat or prevent radiation-induced tissue damage. These medications affect the local tissue RAS, which is present in a variety of tissues. Study of local renal RAS provides further information which might help improve our understanding of radiation-induced renal damage.

1.8.1 Renin-Angiotensin System

The systemic renin-angiotensin system is a complex blood borne hormonal system that has a major role in the regulation of blood pressure. In response to low blood pressure, baroreceptors in the juxtaglomerular system in afferent arterioles of the kidneys release renin. Renin cleaves angiotensinogen which is produced and secreted by liver and converts it into angiotensin I (Ang I). Angiotensin I is then cleaved and converted to the active peptide angiotensin II (Ang II) through the action of angiotensin converting enzyme (ACE). Four types of angiotensin II receptors have been discovered. Functions of angiotensin II type 1 (AT1) receptor and angiotensin II type II (AT2) receptor are well studied. AT2 receptors are more abundant in fetal life and decreases after birth and only present in healthy adults in certain organs like adrenal medulla, uterus and ovaries (18, 19). AT1 receptors are responsible for most of the known physiologic effects of Angiotensin II on a variety of organs; Ang II effects mediated via AT2 receptors in brain includes influencing blood pressure, fluid intake (drinking and salt appetite) and vasopressin secretion. It also affects

cardiovascular system via increasing blood pressure by direct vasoconstriction, stimulation of sympathoadrenal system (centrally and peripherally) and inhibition of vagus nerve effects on the heart. In the kidney, AT1 receptors conduct other effects of Ang II including an increase in the reabsorption of sodium in proximal tubules and induces vasoconstriction (20). It also stimulates the production of aldosterone from adrenal cortex (21).

Aldosterone has a major role in regulating sodium and water reabsorption (potassium excretion) by activating epithelial sodium channels (ENaC) in kidney's distal tubules thus controlling extracellular volume (18).

Besides the systemic RAS, local RAS is found in different organs such as lung, brain and kidney. Although the local RAS system functions are not fully known, research suggests that angiotensin-converting enzyme inhibitors (ACEIs) can clinically attenuate radiation related fibrosis in lungs (22), and cognitive impairment (23, 24) by blocking the renin-angiotensin system. Specifically, administration of ACEIs and AT1 receptor antagonists (AT1RAs) in kidneys prevents radiation nephropathy (25). Studies demonstrate that radiation generally does not affect the renin or Ang II levels (26). Ang II is a potent activator of vascular nicotinamide adenosine dinucleotide phosphate (NADPH) which is a major source for production of reactive oxygen species (ROS) in the vascular system. Local inhibition of renin-angiotensin system (RAS) in kidney which lowers the oxidative stress caused by activity of renin angiotensin system seems to be the underlying cause for the benefits of ACEIs (27).

1.8.2 Kidney

The structural and functional units of kidneys are nephrons. Each nephron consists of two parts. The first part is the renal capsules which are located in the cortex of the kidney. Renal capsule consists of the Bowman's capsule which is a cluster of capillaries (glomerulus) located inside the capsule. Blood is filtered in the glomerulus capillaries to the Bowman's capsule due to the blood pressure. Renal tubule starts from the proximal convoluted tubule (PCT) which is highly coiled and becomes the descending limb of nephron loop. This part of the nephron is followed by ascending limb of the nephron loop and distal coiled convoluted tubule (DTC) which empties into collecting tubes.

There are two types of nephrons: cortical nephrons which consist of 85% of the total renal nephrons and located in the nephron cortex, and juxtaglomerular nephrons. In juxtaglomerular nephrons, Bowman's capsule located at the border of renal cortex and medulla. Their function is important in concentrating urine.

Blood filtration through the Bowman's capsule is passive and depends on the size of the filtrate. Tubular reabsorption of some substances like water, glucose and amino acids are passive and almost complete. Reabsorption of ions are selective and excess ions are allowed to be excreted in urine which has a role in pH adjustment. Tubular secretion actively secrets ions like hydrogen, potassium and nitrogen waste products which also helps in pH regulation and disposal of drugs.

1.8.3 Radiation-Induced Kidney Damage

In recent years, improvements in cancer therapy and significantly higher survival of patients with most types of cancer led to more cases of patients with the long-term adverse

effects of radiation treatment. Kidney is exposed to radiation as a result of different types of radiation treatment like local or whole body radiation. The kidney is highly sensitive to radiation treatment leading to the development of nephropathy at accumulative doses higher than 30 Gy (28).

Radiation nephropathy is a chronic progressive renal dysfunction and may occur within months to years after radiation therapy. Due to its progressive nature, it could be fatal if left untreated. Changes like glomerulosclerosis and tubulointerstitial fibrosis occur due to radiation nephropathy (29).

1.8.4 Radiation Effects on Renin-Angiotensin System

Although it has been reported in some studies (26) that radiation exposure does not affect the expression levels of genes such as angiotensinogen or rennin, genes such as NF-kappaB (NF-κB) is upregulated as the result of radiation exposure (30) and can positively increase the production of angiotensinogen. On the other hand, angiotensin II can stimulate the production of different pro-inflammatory and pro-fibrotic cytokines including activation of NF-κB (31). Therefore, according to preclinical studies treatments such as ACEIs and anti-inflammatory medications can break this cycle and stop the over-activation of RAS and as a result, prevent radiation-induced tissue damage.

1.9 Radiation Impact on Hypothalamic–Pituitary–Adrenal Axis

To our knowledge up to this point, not much information was found on acute effects of radiation on activation of hypothalamic–pituitary–adrenal axis (HPA). The importance of the

HPA axis function in body homeostasis and also great indication of radiotherapy in cancer treatment suggest a great potential for research in this area.

1.9.1 Physiology of Hypothalamic-Pituitary-Adrenal Axis

The nervous system and endocrine system work together to integrate stimuli from the environment and produce proper physiological responses. The nervous system imposes a rapid control via neurotransmitters whereas endocrine system reacts more slowly via secreting and releasing hormones. Hypothalamic-Pituitary-adrenal axis (HPA axis) has a vital role in body's response to stress. Hypothalamus has an important part in regulating body's homeostasis through controlling factors such as fluid volume, temperature and appetite. Hypothalamus is connected to various important parts of the brain; directly to the hippocampus, amygdala and indirectly to brain cortex. In response to different stimuli received by the hypothalamus, it secrets a collection of releasing hormones that reach the anterior pituitary gland through the hypophyseal portal system.

In response to stress, hypophysiotropic neurons localized in the paraventricular nucleus of the hypothalamus (PVN) synthesize and secrete corticotropin-releasing factor (CRF). CRF is a major regulator of HPA axis. It stimulates secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH reaches adrenal cortex via systemic blood circulation and stimulates adrenal cortex to synthesize and secrete corticosteroids like cortisol (32). The physiological effects of cortisol include but not limited to metabolism (stimulating gluconeogenesis and consequently increase in blood glucose levels), maintenance and development of normal immunity, anti-inflammatory, increase blood pressure, vascular tone and activation of the central nervous system, and the critical role of cortisol in stressful

condition and body's survival (33). In addition to all these effects, cortisol also causes the release of catecholamine from adrenal medulla (34).

1.9.2 Adrenal Gland

Adrenal glands are located above the kidneys. Central and cortical parts of the adrenal gland have different origins and secrete different hormones. The adrenal medulla is in the center of the adrenal gland and originated from neural crest tissue. Secretion of hormones in adrenal medulla is directly controlled by the sympathetic nervous system. In fight or flight response to stressors, the adrenal medulla secretes epinephrine. Adrenal cortex has three layers that secrets three hormones two of which are in significant levels. Aldosterone is a mineralocorticoid secreted from adrenal cortex (Zona glomerulosa) through effects of angiotensin II mediated via AT1 receptors (35). Aldosterone increases epithelial sodium channels by increasing its gene expression in distal tubes. As a result, water and sodium reabsorption increases while potassium is secreted into urine. These changes collectively increase the blood pressure. Zona fasciculata in adrenal cortex is responsible for secreting glucocorticoids such as cortisone which increases the blood glucose levels in response to stress through glycogenolysis (glycogen breakdown and production of glucose in liver and muscles) and gluconeogenesis (generation of glucose by degradation of proteins in the liver).

1.9.3 Catecholamine Synthesis Pathway

At the first step of biosynthesis of norepinephrine and epinephrine L-tyrosine is converted to L-dopa by a hydroxyl group added to *meta* position on tyrosine through the action of tyrosine hydroxylase (TH). Tyrosine hydroxylase is a mixed-function oxidase that adds a

hydroxyl group by using molecular oxygen. Next, DOPA decarboxylase (DDC) converts L-dopa to dopamine. Dopamine β-hydroxylase (DBH) is another mixed-function oxidase which then adds a hydroxyl group to the side chain of dopamine leading to the production of norepinephrine (Figure 2). Epinephrine is synthesized by the methyl group transfer from S-adenosylmethionine to norepinephrine through the action of phenylethanolamine N-methyltransferase (PNMT) (36). Increased PNMT expression level is proceeded by increase in transcription factors like Egr-1 (Early growth response 1) and Sp1 (specificity protein 1) in the nucleus. Moreover, stress can induce PNMT levels by transcription factors like glucocorticoid receptor (GR) and Egr-1(37).

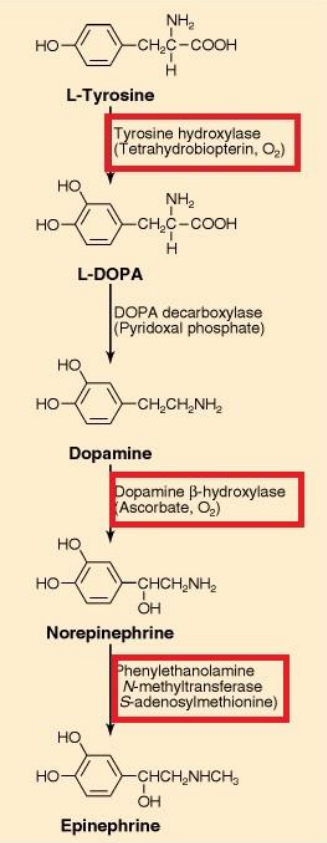

Figure 2 Catecholamine Biosynthesis Pathway (36)

1.10 Antioxidants and Radioprotection

Radioprotection is useful in reducing radiation's toxicity in the tissue after radiotherapy or in accidental exposures such as workplace exposure, war or terrorism (38). Radiation induces a burst of free radicals in the exposed tissue which is responsible for a range of defects. Antioxidants are capable of scavenging those radicals, reducing the DNA damage, cell death and protecting cellular components such as the nucleus, membrane and mitochondria (39).

Multiple dietary supplement (MDS) as showed by Lemon et al. 2003 was developed based on previous studies from a variety of antioxidants and components with scientifically proven efficacy targeting oxidative stress, inflammatory processes, insulin resistance, mitochondrial dysfunction and membrane integrity. Due to these properties MDS was initially developed and proved to be effective in amelioration of age-related cognitive decline (40). It was also proved to be effective in increasing longevity in aging mice model (41), preventing radiation DNA damage by scavenging free radicals (42), improvement in mitochondrial function and ATP production in the brain tissue of aging and mouse model (43).

1.11 Hypothesis

Primary aim of our study was to evaluate the radiation-induced damage. Two organs, kidney and adrenal were selected to address different aspects of radiation-induced effects. We hypothesised that multiple dietary supplement (MDS) which benefits oxidative stress and mitochondrial dysfunction, could attenuate radiation-induced damage. In an animal model (mice) which was chosen to provide a more accurate evaluation of radiation-induced damage

and radioprotective properties of the multiple dietary supplement (MDS) we chose kidney and adrenal tissue to be evaluated.

Considering kidney's vital role in body homeostasis and presence of renal RAS, kidney was selected to be assessed for the radiation induced kidney damage and radioprotective effects of MDS.

We also hypothesized that total body radiation (TBR) may stimulate a response in hypothalamus-pituitary-adrenal (HPA) axis. Adrenal tissue was selected to investigate the radiation-induced stress response and MDS protection against radiation-induced physiological stress by evaluating genes involved in catecholamine biosynthesis pathway.

1.12 Objectives

1 Study Kidney tissue to assess:

1. a Antioxidant response to the radiation-induced oxidative stress and MDS protective effects against radiation-induced oxidative stress.

1. b Radiation-induced DNA damage and cell cycle arrest and MDS protection against radiation-induced DNA damage.

1. c Radiation-induced physiological stress (glucocorticoid release) and MDS protection against radiation-induced physiological stress (glucocorticoid release).

1. d Radiation-induced effects on DNA methyltransferase enzymes and MDS protective effects.

1. e Radiation-induced changes in the renin-angiotensin system activity level and MDS modifying effects on the renin-angiotensin system response to radiation.

2 Study of adrenal tissue to assess:

2. a Antioxidant system response to the radiation-induced oxidative stress and MDS protective effects against radiation-induced oxidative stress.

2. b Radiation-induced DNA damage and cell cycle arrest and MDS protection against radiation-induced DNA damage.

2. c Radiation-induced physiological stress (glucocorticoid release) and MDS protection against radiation-induced physiological stress (glucocorticoid release).

2. d Radiation-induced effects on DNA methyltransferase enzymes and MDS protective effects.

2. e Radiation-induced stress and activation of HPA axis, and MDS protective effects against radiation-induced stress and activation of HPA axis.

1.13 Project Rationale

Studies have shown that dietary supplement can significantly affect five pathways. Since radiation has some similar targets such as mitochondrial function, ROS production, inflammation and membrane disruption MDS could be beneficial in protecting against radiation-induced tissue damage. Since theoretically diet can be effective on some important radiation damage targets we theorized that diet could potentially be used in preventing or treating radiation-induced cellular damage. To provide an accurate view of MDS potential mouse model was chosen.

2.0 Materials and Methods

2.1 Animals

Animal work was done by Dr. J. Lemon at Department of Medical Physics and Applied Radiation Sciences, McMaster University. In this study C57Bl/6J, male mice were used. Care was provided for animals according to the Animal Utilization Protocol as approved by Animal Research Ethics Board. Mice were housed individually and had free access to food and water. They were periodically exposed to a 12 hour light and 12 hour darkness.

2.2 Dietary Supplement

A complex dietary supplement consisting of a variety of vitamins, minerals, herbs and other compounds was previously developed (44). Diet was designed to target major factors in the aging process including oxidative stress, inflammatory processes, insulin resistance and mitochondrial dysfunction. Different elements in the diet were chosen with sufficient scientific evidence of effectiveness on at least one these pathways and the ability to be administered orally. A summary of this information is provided in Table 1.

Dietary supplement components and their target pathways in cells	
Component	**Target in cell**
Vitamin B1	Insulin sensitivity, anti inflammatory
Vitamin B3	Insulin sensitivity, anti inflammatory
Vitamin B6	Insulin sensitivity, anti inflammatory, scavenge O2
Vitamin B12	Insulin sensitivity, anti inflammatory
Vitamin C	Antioxidant in ctosol, scavenge O2,H2O2
Vitamin D	Antioxidant in lipid membrane
Vitamin E	Antioxidant in lipid membrane, scavenge o2,H2O2
Acetyl L-carnitine	Mitochondrial suppport,antioxidant in mitochondria, insulin sensitivity
Alpha lipoic acid	Mitochondrial suppport,antioxidant in mitochondria, insulin sensitivity
ASA	Anti-inflammatory, scavenge NO-
Beta carotene	Antioxidant in lipid membrane, scavenge O2,H2O2
Bioflavonoids	Antioxidant in cytosol and nucleus, scavenge OH-,O2, metal chelator
Chromium	Insulin sensitivity, scavenge H2O2
CoEnzyme Q10	Mitochondrial suppport,antioxidant in mitochondria
Curcumin	Anti infammatory
Folic acid	Antioxidant, maintains glutathione levels, endothelial support
Garlic	Antioxidant in lipid membrane, scavenge O2,H2O2
Ginger	Antioxidant in cytosol and nucleus, scavenge OH-,O2, ONOO-
Ginko biloba	Antioxodant in cytosole, scavenges NO-
Ginseng	Antioxidant in cytosol and nucleus, scavenge OH-,O2, ONOO-
Green tea extract	Antioxodant in cytosole, scavengeS H2O2, OH-
L-glutathione	Enzymatic antioxidant support, antioxidant in cytosol
Magnesium	Insulin sensitivity, cellular support
Melatonin	Antioxidant in cytosol and nucleus, scavenges OH-,H2O2,O-,NO,ONOO-
N-acetyl cycteine	Mitochondrial support, antioxidant in mitochondria
Potassium	Insulin sensitivity, cellular support
Quercitin	Anti-inflammatory
Rutin	Antioxidant in lipid membrane, scavenge OH-,O2, metal chelator
Selenium	Scavenges H2O2, enzymatic antioxidant support, insulin sensitivity

Table 1: Components of multiple dietary supplement and their respective cellular targets

2.3 Feeding

Doses of dietary supplement for mice was determined by human recommended dosage. Mice received ten times of recommended dose of supplement for their weight to compensate for higher metabolism rate in mice compared to humans. Dietary supplement was soaked into bagel bits. It was made sure that entire bagel bits were consumed each time. However, any left pieces were recorded during the feeding process. Dietary supplement was fed on regular basis pre and post-radiation according to the groups assigned. To maintain a healthy and

sufficient diet and avoid the risk of malnourishment mice body weight were recorded twice a week.

2.4 Irradiation

Awake animals were immobilized in a polyvinyl chloride (PVC) tubes 30 minutes before irradiation. Treatment groups including radiation received 5 Gy whole body radiation from a cesium 137 (energy 662 KeV) source (Taylor cesium source) as they were immobilized in plastic tubes and had rotation around the coronal/sagittal planes but not transverse plane. Mice in the control group were subjected to the same condition but they were shielded from radiation by custom build lead shielding which was used to protect the body from irradiation.

2.5 Treatment Groups

Mice were divided randomly into five different groups. Animal's age was between 9 to 11 weeks old and they received radiation a month after study initiation. To accurately compare different treatments, five groups were considered as it is shown in ***Table 2***. The control group did not receive any supplement and underwent sham radiation. They were placed in proper tubes, kept in the same radiation chamber for the same amount of time as radiation-treated mice without receiving any radiation. The second group (MDS) received the dietary supplement for the duration of study starting from a month before radiation date. The third group (5 Gy) consists of animals receiving 5 Gy radiation from a cesium source. Last two groups were assigned to determine the effectiveness of multiple dietary supplement to prevent the radiation-induced tissue damage. Supplement+ whole body 5 Gy irradiation (MDS-5 Gy-MDS) group received daily supplements for 30 days before radiation and

continued to receive the supplement daily until the end of the study. 5 Gy whole body irradiation+ supplement (5 Gy-MDS) group started to receive daily supplement after receiving 5 Gy radiation. From now on the short-forms mentioned above will be used. Animals were sacrificed 2, 30 and 120 days after 5 Gy radiation treatment. Organs were harvested and flash frozen for further analysis.

Group ID	Treatments	Mice ID# (Tissues harvested 2d/30d/120d post-irradiation)		
		2 d	30 d	120 d
I	**Control (no treatment)**	1 – 5	6 – 10	11 – 15
II	**Supplement only (MDS)** -supplement fed for duration of study -sham irradiated 30d after start of supplement	1 – 5	6 – 10	11 – 15
III	**5 Gy whole body irradiation (5 Gy)**	1 – 5	6 – 10	11 – 15
IV	**Supplement+5 Gy whole body irradiation (MDS+5 Gy+MDS)** -supplement fed for duration of study -Irradiated 30d after start of supplement	1 – 5	6 – 10	11 – 15
V	**5 Gy whole body irradiation + supplement (5 Gy+MDS)** -fed supplement for the duration of study after irradiation	1 – 5	6 – 10	11 – 15

Table 2: Study's treatment groups and associated information

2.6 mRNA Extraction

Approximately 50 mg of flash frozen tissue was weighed and placed in round bottom autoclaved Eppendorf microcentrifuge tubes. 1 ml TRIzol (TRI reagent, Sigma®) was added to each tube with one Diethylpyrocarbonate (DEPC, Amersco®) treated stainless steel bead. Tubes were placed in Tissue lyser for 2 cycles at 30 Hz for 2 min. The homogenized tissue was transferred to another tube and centrifuged at 12,000 x g for 10 min at 4˚C. The supernatant was then transferred to a fresh tube. 200 µl of chloroform per 1 ml of TRI reagent was added to the sample and mixed well. The samples were incubated at room temperature for 10 min.

Tubes then were centrifuged at 12,000 x g for 10 min at 4˚C. After centrifugation, the aqueous phase which contains the RNA (ribonucleic acid) was transferred to a fresh tube while the DNA and protein found in the interphase and lower phase were discarded. 500 µl of isopropanol was added per 1 ml of TRI reagent, and each tube was vortexed for 10-15 sec and incubated at room temperature for 10 min. The tubes were then centrifuged at 12,000 x g for 8 min at 4℃ and supernatant was discarded afterwards. The pellet was suspended in 1 ml of 70% ethanol per tube by flicking the tube. The tubes were then centrifuged at 7,500 x g for 5 min and the ethanol was removed. Pellets containing purified RNA were subsequently air dried briefly and dissolved in DEPC-treated water (30 µl) by incubating the tubes for 10 min at 37 ˚C. For long term storage RNA samples were stored at -80˚C. The RNA samples were analysed using NanoDrop (ND-1000 spectrophotometer) to measure content and purity of RNA which was evaluate by the ratio of absorbance at 260 nm and 280 nm (2 or higher is pure for RNA) and 260 nm and 230 nm (2-2.2 is pure for nucleic acid).

2.6.1 RNA Integrity

1% agarose gel was prepared by adding 1 g agarose to 100 ml of 1X Tris-borate (1.08 g Tris-base, 0.55 g boric acid, 0.4 ml of 0.5 M EDTA pH 8.0) electrophoresis buffer. The mixture was microwaved until agarose was completely dissolved. 10mg/ml ethidium bromide (EtBr) was added to the cooled 1% agarose gel solution. The mixture was poured into a prepared tray and was allowed to solidify for an hour. RNA samples were prepared by mixing 500 ng of total RNA with 2 µl of 6X gel loading dye (30% Glycerole,0.25% Bromophenol Blue) and nuclease-free water to a total volume of 12 µl. 1 kb ladder (Invitrogen®) was used for reference. Gel was run at 90 V until band separation was achieved.

To visualize the RNA bands, Bio-Rad agarose gel imaging Chemi-Doc (software: Quantity One) was used.

2.7 Generation of cDNA from mRNA

2.7.1 DNase Treatment

2 µl 10X DNase reaction buffer (DNase kit from Sigma®) and 2 µl DNase I (1 u/ µl) were added to tubes containing 2 µg of RNA sample. The final volume of the reaction was adjusted to 20 µl with DEPC-treated water. Tubes then incubated at room temperature for 15 min. 2 µl of stop solution (Sigma®) was immediately added to the reaction and heated at 70 ˚C for 10 min. The samples were then chilled on ice.

2.7.2 Reverse Transcription

1 µg (1 µl) of random primers was added (0.5 µg of primers per 1 µg of mRNA) to the DNase-treated RNA samples. Tubes were then sealed, flicked, spun down and immediately chilled on ice. 27 µl of the Master Mix containing (12.5 µl DEPC water, 10 µl M-MuLV 5X buffer (Promega®), 2.5 µl mixed dNTPs and 2 µl M-MuLV reverse transcriptase (Promega®) was then added to the tubes. For the preparation of control negatives samples, master mix was prepared without M-MuLV reverse transcriptase. Tubes were sealed, flicked, spun down and incubated for 60 min at 37 ˚C.

2.8 Quantitative PCR (qPCR)

2.8.1 Primers Design

To design primers for each gene first, the sequence of the target gene was obtained from NCBI. Primer3 software was used to design primers. Messenger RNA (mRNA) sequence was uploaded in the software. Software's criteria to select primers from numerous other possibilities included primer length between 18 – 22 base pairs, optimal GC content between 40%-60% (to optimize the melting temperature), melting temperature between 57-63 degrees and checking primers sequence for the possibility of RNA secondary structures like hairpins and dimers. The software also checked for other non-specific PCR products. List of primers that has been used in this study is provided in Table 3.

We also obtained some of the primer's sequences from the primer bank. For this database, NCBI Gene ID was used. GenBank provided a list of primers which had been tested extensively by qPCR experiments for specificity and efficiency.

Gene name	Primer sequence	Efficiency	Gene name	Primer sequence	Efficiency
Angiotensinogen	TCTCCTTTACCACAACAAGAGCA CTTCTCATTCACAGGGGAGGT	102.50%	CDKN1A	GACCTGGGAGGGGACAAGAG AGACACCAGAGTGCAAGAC	94.85%
Renin	CTCTCTGGGCACTCTTGTTGC GGGAGGTAAGATTGGTCAAGGA	105.80%	GADD45A	GAAAGGATGGACACGGTGGG GGGTCTACGTTGAGCAGC	105.56%
ACE	AGGTTGGGCTACTCCAGGAC GGTGAGTTGTTGTCTGGCTTC	98.63%	γH2AX	GCCTCTCAGGAGTACTGAGGG CCCGAAGTGGCTCAGCTCTTT	110.73
AgtR1A	AACAGCTTGGTGGTGATCGTC CATAGCGGTATAGACAGCCCA	96.96%	Per2	ACAAGAAGGCCAAGGGGAAGG GGCTCTCACTGGACATTAGCAG	95.68%
PNMT	CAGACCTGAAGCACGCTACAG TAGTTGTTGCGGAGATAGGCG	89.10%	DDB2	ACCGA(ACCGA(ACCGAGTACGTC CTTGG(CTTGG(CTTGGCTTCGGG	97.81%
TH	GCCGTCTCAGAGCAGGATAC AGCATTTCCATCCCTCTCCT	95.90%	NRF2	CAGCACATCCAGACAGACACCA TGGGAATGTCTCTGCCAAAAGCT	99.79%
DBH	AATCTGCAGCCTTTGCCTAA AGGAGGAGGAGGGTCTGAAG	104.60%	Catalase	CCTGACATGGTCTGGGAC CCATAGCCATTCATGTGCCG	100.25%
Egr-1	GACGAGTTATCCCAGCCAAA GGCAGAGGAAGACGATGAAG	94.29%	GPx1	GGTGCTGCTCATTGAGAATGTCG GGGAAACCGAGCACCACCAG	91.57%
Sp-1	TCATGGATCTGGTGGTGATGGG GCTCTTCCCTCACTGTCTTTGC	100.40%	NOS3	TTTGCTGCCCTTGGCCTGCG CTCTGAACTCATGTACCAGCCG	89.57%
Ap-2	CAATGAGCAAGTGGCAAGAA AGGGCCTCGGTGAGATAGTT	105.58%	SOD1	CGGATGAAGAGAGGCATGTTGGA CAATGATGGAATGCTCTCCTGAG	103.37%
RPL29	ACATGGCCAAGTCCAAGAAC TGCATCTTCTTCAGGCCTTT	94.33%	SOD2	TTACGACTATGGCGCGCTGGA TCGTGGTACTTCTCCTCGGTG	93.54%
Beta Actin	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT	94.26%	SOD3	GGCCTGTGGCTCTGTCACC CCTATCTTCTCAACCAGGTCAAG	94.92%
HSD11β1	GAAGAGTTCAGACCAGAAATGCT GAAGAGTTCAGACCAGAAATGCT	90.64%	DNMT1	AAGAATGGTGTTGTCTACCGAC CATCCAGGTTGCTCCCCTTG	102.60%
GR	AGGCCGCTCAGTGTTTTCTA TACAGCTTCCACACGTCAGC	103.60%	DNMT3A	GAGGGAACTGAGACCCCAC CTGGAAGGTGAGTCTTGGCA	108.30%
			DNMT3B	AGCGGGTATGAGGAGTGCAT GGGAGCATCCTTCGTGTCTG	111.30%

Table 3: Primer sequence of targeted genes in kidney and adrenal tissue and their respective efficiency.

2.8.2 Primer Validation

To validate primers for qPCR analysis, a serial dilution of cDNA samples containing 30, 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 ng cDNA was prepared. To prepare the final mixture 10 µl Quanta mix, 2.6 µl ddH2O, 1.2 µl 600 nM forward and 1.2 µl 600 nM reverse primer was added to 5 µl of cDNA with concentrations mentioned above. qPCR machine determined the initial complementary DNA (cDNA) level in samples by providing cycle threshold (Ct) value. Ct value or cycle threshold is the number of polymerase chain reaction (PCR) cycles needed for fluorescence level to cross the threshold of the machine. The higher Ct value, the lower initial cDNA level in the sample.

Standard curves were generated in Excel by blotting log cDNA concentration on x-axis and Ct values on Y-axis. According to the properties of the curve including determining the R2 (coefficient of correlation) and efficiency value were evaluated. Efficiency considered desirable between 90%-110%. Melting temperature was also evaluated for each primer to determine whether primer generated a single PCR product. In case a primer did not meet the criteria other primers had been designed and tested in the same manner to provide same quality assay for every gene screened.

2.8.3 qPCR

Frozen cDNA samples were thawed and diluted by DEPC treated RNase-free water to 2 ng/ µl concentration. Each reaction contained 5 µl of cDNA sample and 15 µl of a master mix containing target gene's primers (600 nm), Quanta q-PCR mix and DEPC treated water to the total volume of reaction 20 µl. Accumulation of fluorescence signals from CYBR green (PerfeCta®SYBR® Green FastMix®, Quanta Biosience®) incorporated into double strand DNA during PCR was detected and analyzed by BIO-RAD Chromo4 Real PCR machine.

For q-PCR, BIO-RAD Chromo4 Real PCR machine was used (Software: Option Monitor 3). qPCR settings: q-PCR microtubes were placed in the machine. Following setting was used to run each experiment. Starting at 95˚C for 2 min, at 95 ˚C for 30 seconds (DNA denaturation), at 59 ˚C for 30 seconds (annealing temperature is specific for each primer set), at 72 ˚C for 30 sec (extension), plate read and then cycles starting from incubation at 95 ˚C for 30 sec repeated for 40 times.

2.9 qPCR Data Analysis

Machine recorded Ct value as a cycle number that fluorescence intensity of qPCR reaction reached the pre-set threshold (0.01). This information was plotted as Ct on x-axis and Fluorescence intensity on the y-axis. CYBR green in Quanta mix was used as a source of fluorescence detectable by qPCR machine. The dye gets attached to double strand DNA. To compare the initial amount of DNA in each sample as an indicator of mRNA level, Ct values were evaluated for each sample, initial for two internal reference genes and then for each target gene. After each q-PCR, all data was collected using Ct threshold of 0.01 and copied in the Excel for further analysis

The Δct mathematical model was applied for qPCR data analysis. Two reference genes including β actin and RPL29 were analyzed for each sample. GEOMEAN of these two values were used in calculations. To analyze the data first ΔCt ($=Ct_{sample}-Ct\ r_{eference}$) for each sample, an average of ΔCt for all samples and Standard Error of Means (SEM) were calculated.
The $\Delta ct_{average}$ was converted to $2^{-\Delta Ct\ average}$. To plot the final data, up and down errors needed to be individually calculated as follow; Error-Up ($2^{-[\Delta Ct\ average-SEM]}$)- $2^{-\Delta Ct\ average}$ and Error-Down ($2^{-\Delta Ct\ average}$-($2^{-[\Delta Ct\ average+S.E.M]}$). The $2\text{-}\Delta c^{t\ average}$ was plotted with Error-Up and Error-Down (45).

2.10 Statistical Analysis

SPSS version 18 was used for data analysis. For data analysis, One-way ANOVA with Tuckey's post-hoc was utilized. Differences considered significant if post-hoc Tukey test<0.05.

3.0 Kidney

3.1.1 Introduction

Exposure of biological systems to ionizing radiation causes tissue and ultimately organ damage. Involvement of local RAS system in radiation injury has been previously established in tissues like kidney and brain (46, 47). Although according to literature RAS is not upregulated its physiological function aggravates the oxidative stress caused by radiation (31). We hypothesized that MDS protects the tissue from radiation-induced tissue damage due to its protective effects on mitochondria, genome and free radical scavenging properties. To address this hypothesis important genes in RAS, antioxidant enzymes, physiological stress response, markers of DNA damage and DNA repair and DNA methyltransferase enzymes were selected.

3.1.2 Objectives

To assess kidney for:

A) Radiation-induced changes in expression level of genes involved in the cellular antioxidant system, DNA repair, cell cycle progression, DNA methyltransferase enzymes and markers of physiological stress and the renin-angiotensin system.

B) The expression level of the above genes in groups receiving MDS in combination with radiation.

3.2 Results

The results from the genes studied in kidney tissue including cellular antioxidant system, DNA damage repair, cell cycle progression, RAS, markers of cellular physiological stress response and DNA methyltransferase family, are summarized in Table 4.

		mRNA Expression Changes Compared to Control											
		2 Day				30 Day				120 Day			
	Gene	MDS	5 Gy	MDS 5Gy MDS	5 Gy MDS	MDS	5 Gy	MDS 5Gy MDS	5 Gy MDS	MDS	5 Gy	MDS 5Gy MDS	5 Gy MDS
Cellular anti-oxidant system	NRF2 - nuclear factor 2	↔	↑	↑		↔	↔	↔					
	Catalase	↔	↑	↑		↔	↔	↔					
	GPX1-glutathione peroxidase 1	↔	↑	↔		↔	↑	↔					
	NOS3 - endothelial nitric oxide synthase	↔	↑	↔		↔	↔	↔					
	SOD1 – superoxide dismutase 1	↔	↔	↓		↓	↓	↓					
	SOD2 – superoxide dismutase 2	↔	↔	↓		↔	↑	↔					
	SOD3 – superoxide dismutase 3	↔	↔	↔		↔	↑	↑					
DNA damage repair/ cell cycle progression	CDKN1A – cyclin-dependent kinase inhibitor 1	↔	↑	↑		↔	↑	↑					
	GADD45 – Growth arrest and DNA-damage-inducible protein	↔	↔	↔		↔	↑	↔					
	H2AX – H2A histone family, member X	↑	↑	↔		↑	↑	↑					
	PER2 –period circadian clock 2	↔	↑	↔		↔	↔	↔					
RAS system	Agt – angiotensinogen	↔	↔	↓	↓	↔	↔	↓	↓	↔	↔	↓	↓
	ACE-angiotensin converting enzyme	↔	↔	↓	↔	↔	↔	↔	↑	↔	↔	↔	↔
	Ren1-renin 1	↔	↓	↓	↔	↔	↔	↔	↔	↔	↔	↔	↔
	AgtR1A-angiotensin II receptor 1 A	↔	↔	↔	↔	↔	↔	↔	↓	↔	↔	↔	↔
	Sp1-trans-acting transcription factor	↔	↔	↔	↔	↔	↔	↔	↓	↔	↔	↔	↔
Physiological stress	HSD11β1 – 11β-hydroxysteroid dehydrogenase type 1	↔	↓	↓		↔	↔	↔					
	Nrc1-glucocorticoid receptor	↔	↔	↔		↔	↔	↓					
DNA methyl-transferase	DNMT1-DNA methyltransferases	↔	↔	↔	↔	↑	↔	↔	↔	↑	↔	↔	↔
	DNMT3A-DNA methyltransferases	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
	DNMT3B-DNA methyltransferases	↔	↔	↔	↔	↔	↓	↔	↔	↔	↔	↔	↔

Table 4 Kidney mRNA expression Analysis Summary. mRNA Expression Changes Compared to Control (Group#1), ↔=no change; ↓=decrease; ↑=increase

3.2.1 Kidney Local Renin-Angiotensin System

The renin-angiotensin system is a major contributor to radiation-induced oxidative tissue damage. Irradiation-induced changes in RAS system at 2, 30 and 120 days post-irradiation in kidney tissue were analyzed by measuring mRNA transcript levels. Angiotensinogen is the primary precursor for the production of Ang II which has a significant role in radiation-induced tissue injury by producing reactive oxygen species via NADPH-oxidase enzyme activity. 5 Gy whole body radiation did not cause any significant change in the expression

level of angiotensinogen in the kidney tissue compared to the control group. However, in the MDS-5 Gy-MDS group, the angiotensinogen expression levels decreased to 1.4, 1.47 and 3.2-fold compared to the control, 2, 30 and 120 days post-radiation exposure. In addition, in the 5 Gy-MDS group the angiotensinogen expression level also significantly decreased by 1.17, 2.44 and 2.6-fold compared to the control at 2, 30 and 120 days post-radiation. MDS demonstrated the ability to decrease angiotensinogen expression level in MDS-5 Gy-MDS and 5 Gy-MDS groups. Decreased angiotensinogen expression level potentially lead to a decrease in the production of angiotensin II, the decreased activity level of local renal RAS and consequently lowers production of ROS (Figure 3A).

Angiotensin-converting enzyme (ACE) converts Ang I to Ang II. mRNA expression analysis of ACE showed no change in expression level due to 5 Gy whole body radiation at 2, 30 and 120 days post-radiation. In MDS-5 Gy-MDS group, ACE expression level showed an initial decrease (2 days) of 1.7-fold compared to the control. Moreover, 5 Gy-MDS group showed a significant increase in the expression level of ACE to 1.3-fold of the control group at 30 days post-radiation (Figure 3B).

Renin is a proteolytic enzyme responsible for converting angiotensinogen to Ang I. 5 Gy whole body radiation caused a significant decrease in renin mRNA expression to 1.4-fold of the control group 2 post-radiation. However, renin expression level did not show any significant changes compared to control at other time points. MDS-5 Gy-MDS also showed a significant 2.24 -fold decrease in renin expression level compared to the control 2 post-radiation (Figure 3C).

AgtR1A codes for Ang II receptor type 1, which mediates the Ang II biological effects via the function of NADPH oxidase enzyme and production of ROS as a signaling messenger. 5 Gy whole body radiation did not change the expression level of AgtR1A in kidney tissue. MDS-5Gy-MDS group also showed no significant change compared to the control. However, AgtR1A expression level was significantly downregulated in 5 Gy-MDS group to 1.7-fold compared to the control group 30 days post-radiation. MDS potential to lower the expression level of AgtR1A at 30 days post-radiation, could potentially lead to decreased ability of angiotensin II to produce its biological effects (Figure 3D).

Sp1 (specificity protein 1) transcription factor is important in the regulation of renin expression levels. mRNA expression analysis of Sp1 demonstrated that 5 Gy whole body radiation caused no change in expression level if Sp1 compared to the control at any time point. On the other hand, 5Gy-MDS group demonstrated a significant 1.45-fold decrease in expression level of Sp1 compared to the control group 30 days post-radiation (Figure 3E).

Taken together, the data from mRNA expression levels suggest MDS potential, to reduce the renin-angiotensin system function, mainly by persistent downregulation of angiotensinogen gene as the precursor through the time points and also by downregulation of ACE, renin, AgTR1A and SP1 at some of the time points. This finding supports MDS potential to be used in preventing or treating radiation tissue damage in kidney tissue.

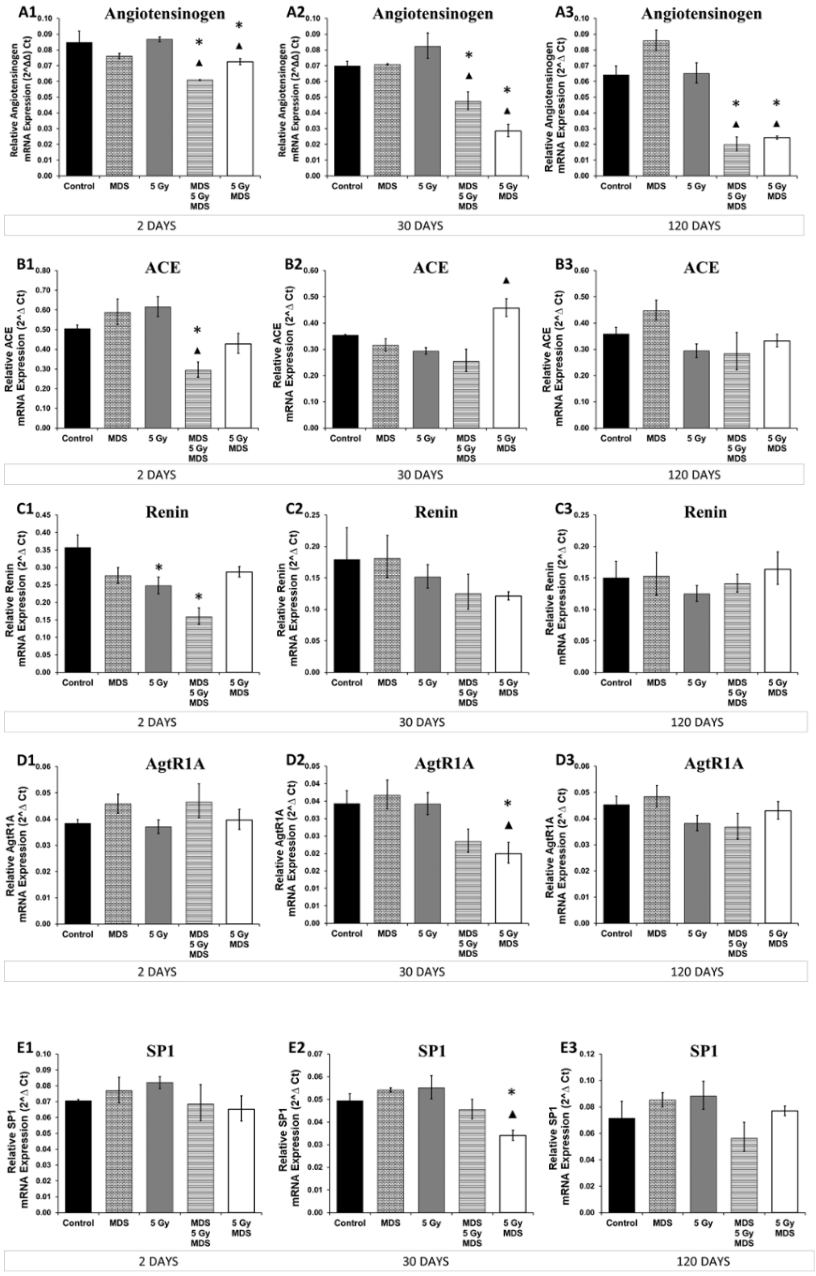

Figure 3 RT-qPCR mRNA expression analysis of markers of Renin-Angiotensin system in 2, 30 and 120 days post-irradiation (or sham-irradiated) kidney tissue. mRNA expression of Angiotensinogen (panel A), angiotensin converting enzyme (ACE, panel B), Renin (panel C)), Angiotensin II Receptor type 1 (AgtR1A, panel D), trans-acting transcription factor (Sp1; panel E) were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA.

3.2.2 Oxidative Stress and Antioxidant Enzymes

Irradiation-induced oxidative stress in kidney was assessed by measuring the changes in transcription levels of antioxidant enzymes. Nuclear factor like-2 (NRF2) encodes an important transcription factor for regulating transcription of antioxidant genes. RT-qPCR analysis showed a significant 2.6-fold increase in expression levels of NRF2 in 5 Gy radiation group compared to the control 2 days post-radiation. In the MDS-5 Gy-MDS group expression level of NRF2 was elevated to 2.8-fold compared to the control group 2 days post-radiation. NRF2 expression levels returned to the control level by 30 days post-radiation in both groups (Figure 4A). NRF2 upregulation was induced by 5 Gy radiation-induced oxidative stress and in the MDS-5 Gy-MDS group, MDS did not change the tissues response.

Catalase and glutathione peroxidase 1 (GPX1) were studied to evaluate the cellular response to increased levels of hydrogen peroxide. RT-qPCR analysis of catalase gene in the 5 Gy and MDS-5 Gy-MDS groups, demonstrated a similar trend of upregulation of the catalase gene expression by 2.25 and 2.3-fold compared to the control respectively 2 days post-radiation (Figure 4B). Catalase expression levels were sensitive to 5 Gy radiation in kidney tissue and MDS did change this response.

RT-qPCR analysis of GPX1 gene in kidney demonstrated a distinct pattern of expression between 5 Gy and MDS-5 Gy-MDS groups. In the 5 Gy irradiation group, GPX1 expression levels raised significantly to 2.3 and 1.4-fold compared to the control group, 2 and 30 days post-radiation. However, in MDS-5 Gy-MDS, MDS protected kidney tissue from the irradiation-induced oxidative stress as GPX1 expression levels showed no significant increased compared to the control (Figure 4C).

NOS3 catalyzes the production of NO in the cytoplasm and due to NO uncoupling causes cellular damage. RT-qPCR analysis of NOS3 gene in kidney tissue showed significant 1.7-fold increase in enzyme expression level in the 5 Gy group compared to the control 2 days post-radiation. MDS effectively protected the kidney tissue from radiation-induced oxidative stress in such a way that no upregulation in NOS3 expression level as observed in the MDS-5 Gy-MDS group 2 and days post-radiation (Figure 4D).

Kidney tissue analysis for SOD1 gene showed in the 5 Gy group SOD1 expression levels remained unchanged compared to the control 2 days post-radiation, but it was significantly decreased to 1.3-fold of the control, 30 days post-radiation. In MDS-5 Gy-MDS group SOD1 expression levels significantly decreased to 1.5 and 1.6-fold of the control in 2 and 30 days post-radiation. Also, MDS was able to reduce SOD1 expression levels significantly by 30 days post-radiation (Figure 4E). Collectively it is suggested that SOD1 expression level is regulated according to superoxide levels in MDS and MDS-5 Gy-MDS group which indirectly indicate that kidney was protected by MDS from radiation-induced cytoplasmic superoxide.

RT-qPCR analysis showed that 5 Gy radiation resulted in increased expression levels of SOD2 at 30 days post-radiation. On the other hand, the MDS-5Gy-MDS group showed decreased expression levels of SOD2 to 1.25-fold of the control group 2 days post-radiation (Figure 4F). Data from the SOD2 mRNA expression level indirectly suggest that MDS protected the mitochondria of the kidney tissue from radiation-induced superoxide production. SOD3 expression levels were also evaluated but due to very low expression levels the results have not been presented.

Taken together data suggest that MDS-5Gy-MDS group was protected from radiation-induced oxidative stress and consequently upregulation of GPX1, NOS3 and SOD2 did not happen.

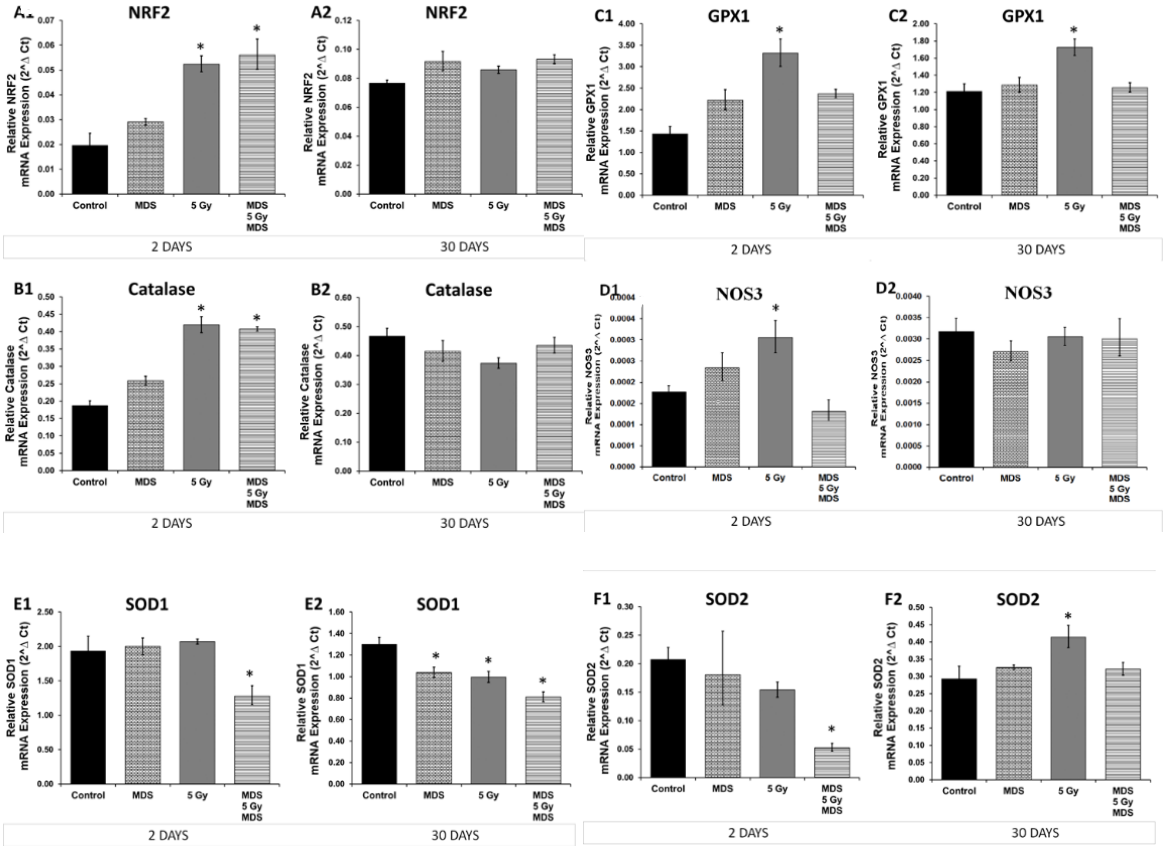

Figure 4RT-qPCR mRNA expression analysis of markers of endogenous antioxidants system at 2 and 30 days post-irradiation (or sham-irradiated) kidney tissue. mRNA expression of nuclear factor-like 2 (NRF2; panel A), catalase (panel B), glutathione peroxidase 1 (GP X1; panel C), endothelial nitric oxide synthase (NOS3, panel D), superoxide dismutase 1 (SOD1; panel E), superoxide dismutase 2 (SOD2; panel F), were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc (73) analysis. (*; significant data compared to control).

3.2.3 Cell Cycle Progression and DNA Repair Enzymes

To determine MDS's radioprotective effects on DNA genes listed below has been studies. CDKN1a (cyclin-Dependent Kinase Inhibitor 1A) encoded a protein that inhibits the function of cyclin-CDK2 in response to a variety of stress stimuli which causes G1 phase arrest and also regulates DNA repair. 5 Gy radiation caused a 9-fold increase in CDKN1a expression levels compared to the control 2 days post-radiation. CDKN1a expression levels remained elevated by 4.6-fold compared to the control 30 days post-radiation. Elevated expression levels of CDKN1a in kidney tissue suggested cell cycle arrest due to 5 Gy radiation. Similarly, the MDS-5 Gy-MDS group showed 8.2 and 3.8-fold increase in CDKN1A expression levels compared to the control 2 and 30 days post-radiation respectively. MDS did not protect the kidney tissue from irradiation-induced cell cycle arrest (Figure 5A).

GADD45 (Growth Arrest and DNA Damage) protein is induced by stress-induced growth arrest and DNA damage and it is important in DNA base damage repair. 5Gy irradiation resulted in a 2-fold increase in GADD45 mRNA expression levels compared to the control 30 days post-radiation. No significant change in expression levels of GADD45 was observed in MDS-5Gy-MDS group compared to the control (Figure 5B). Study of gene expression levels suggested that MDS protected the DNA in kidney tissue from radiation-induced DNA damage.

H2AX is a member of the family of histone proteins which is very important in signaling DNA damage and DNA repair. Phosphorylation on a specific serine at the C-terminal of H2AX, which causes activation of protein is considered to be a major sign of DNA damage. RT-qPCR analysis of kidney tissue 2 and 30 days after post-radiation showed 5 Gy whole

body radiation resulted in a 2 and 3.8-fold increase compared to the control group. MDS increased the basic expression levels of H2AX expression levels in MDS group by 2 and 2.5-fold compared to the control group. MDS also modified the kidney cellular response to 5 Gy radiation as MDS-5 Gy-MDS group did not show any significant increase in H2AX expression levels at 2 days which was followed by 2.3-fold increase compared to the control 30 days post-radiation (Figure 5C).

Period circadian clock 2 (PER2) belongs to a family of genes which regulates DNA damage response including DNA repair. mRNA expression analysis of PER2 demonstrated 3.3-fold increase 2 days post-radiation in kidney tissue compared to the control. This finding illustrates a significant activation of cellular DNA damage response after 5 Gy whole body radiation. The MDS-5 Gy-MDS group, however, demonstrated no change in PER2 expression levels compared to the control (Figure 5D). MDS successfully protected DNA from 5 Gy radiation-induced DNA damage.

Collectively MDS demonstrated the ability to protect kidney tissue from radiation-induced DNA damage as it was demonstrated by the lack of upregulation of DNA repair markers such as GADD45A and PER2.

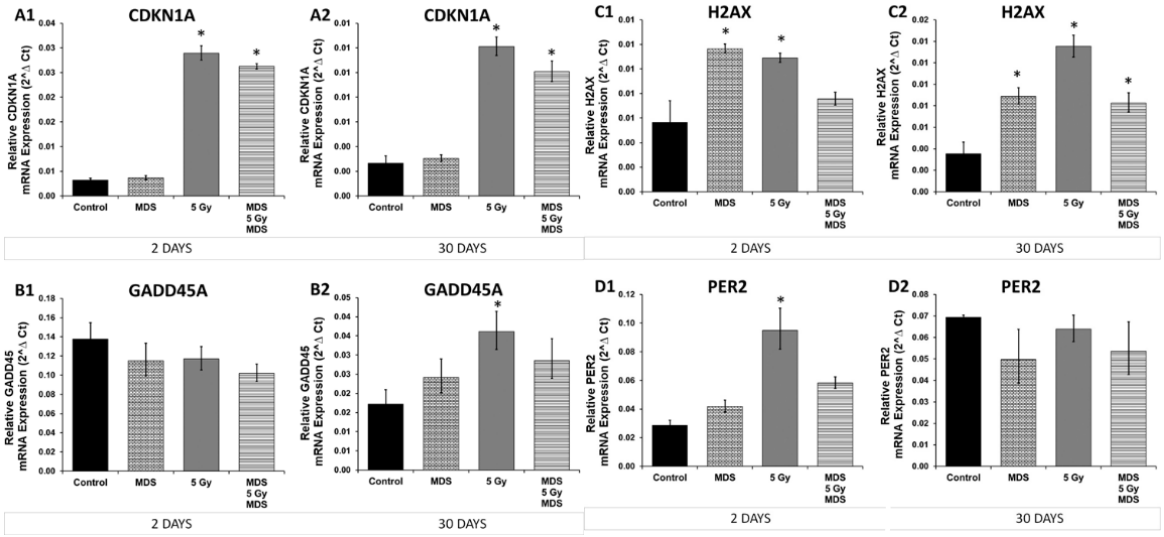

Figure 5 RT-qPCR mRNA expression analysis of markers of DNA damage/repair and cell cycle progression in 2 and 30 days post-irradiation (or sham-irradiated) kidney tissue. mRNA expression of cyclin-dependent kinase inhibitor 1 (CDKN1A; panel A), Growth arrest and DNA-damage-inducible protein (GADD45; panel B), H2A histone family (H2AX; panel C), period circadian protein 2 (PER2; panel D), and were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA allowed by Tukey's post-hoc analysis. (*; significant data compared to control).

3.2.4 Physiological Stress Response

Glucocorticoids are secreted in response to stressors and distribute in the body tissues. At the cellular level, their level of action is determined by factors such as HSD11B1 (11β hydroxysteroid dehydrogenase) and glucocorticoid receptors (GR). HSD11B1 act as a reductase enzyme which causes intracellular metabolism of glucocorticoids and converts the inert cortisone to active cortisol. Therefore expression levels of HSD11B1 and GR were studied to evaluate cellular utilization of glucocorticoids and activation of stress signaling in animals following 5 Gy radiation. RT-qPCR analysis of HSD11B1 demonstrated a 2-fold decrease in the 5 Gy group compared to the control at 2 days post-radiation. MDS-5 Gy-MDS group also showed a significant 8-fold decrease in HSD11B1 expression levels compared to the control group (Figure 6A).

5 Gy whole body radiation did not cause any change in GR (Glucocorticoid receptor) expression levels compared to the control. However, MDS-5 Gy-MDS group showed a significant 1.4-fold decrease in GR expression levels compared to the control (Figure 6B).

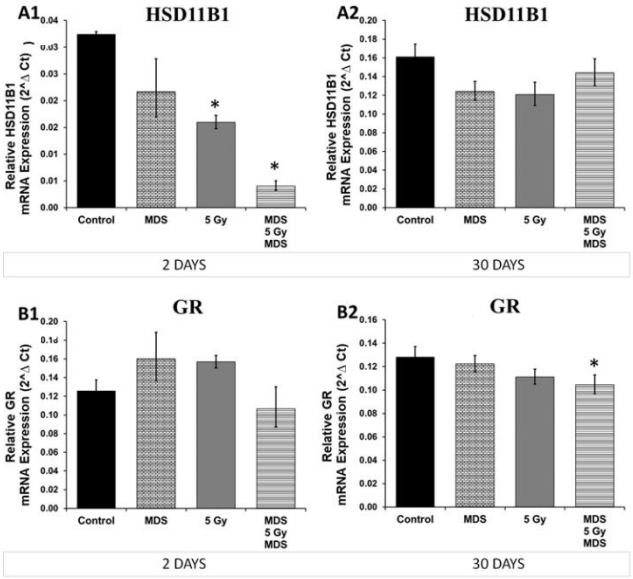

Figure 6 RT-qPCR mRNA expression analysis of markers of physiological stress response in 2 and 30 days post-irradiation (or sham-irradiated) kidney tissue. mRNA expression of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1; panel A), nuclear receptor subfamily 3, group C, member 1 or Glucocorticoid receptor (NR3C1; panel B) were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc analysis. (*; significant data compared to control).

3.2.5 DNMT Family

mRNA expression levels of DNA methyltransferase family (DNMTs) genes which code for three important enzymes responsible for maintenance and *de novo* DNA methylation have been studied. 5 Gy whole body radiation caused no significant change in mRNA expression levels of DNMT1 (DNA methyltransferase 1) in kidney tissue 2, 30 and 120 days post-radiation. Interestingly, MDS group demonstrated a significant 1.5 and 1.27-fold increase in DNMT1 expression levels compared to the control 30 and 120 days post-radiation (Figure 7A).

mRNA expression levels of DNA methyltransferase 3A (DNMT3A) demonstrated no significant change compared to the control caused by 5Gy whole body radiation. Similarly, MDS, did not affect the DNMT3A expression levels in any of the groups receiving MDS, alone or in combination with 5 Gy whole body radiation (Figure 7B).

Study of mRNA expression levels of DNA methyltransferase 3B (DNMT3B) which is known for de novo DNA methylation (creation of new methylation marks on the DNA) showed that 5 Gy whole body radiation caused a significant 5.7-fold decrease in mRNA level compared to the control group 30 days post-irradiation. In MDS-5Gy-MDS and 5Gy-MDS, however, MDS protected the kidney tissue from radiation-induced downregulation of DNMT3B, which was demonstrated by the lack of any significant change in DNMT3B expression levels compared to the control (Figure 7C).

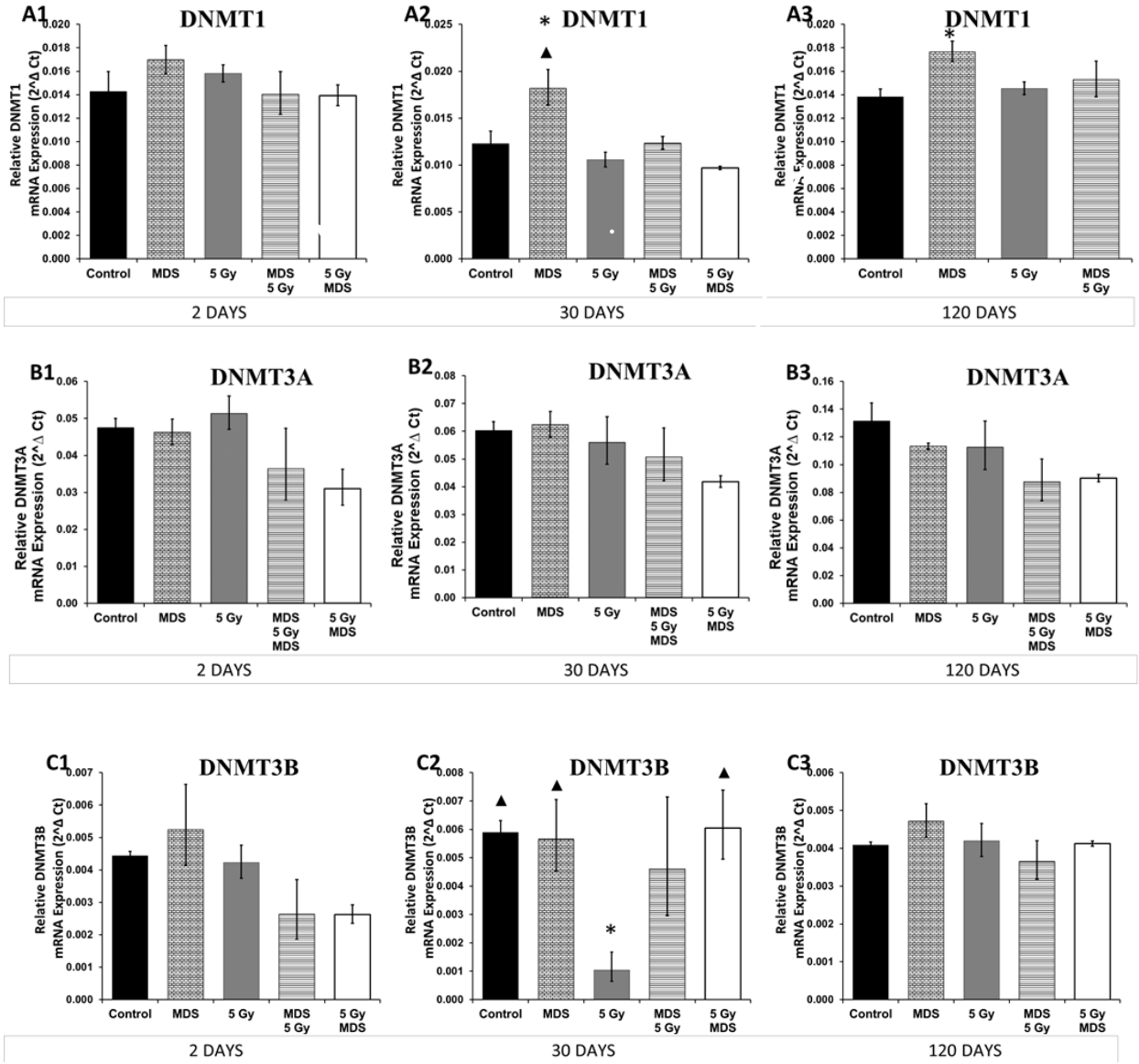

Figure 7 RT-qPCR mRNA expression analysis of markers of DNA methylation in 2,30 and 120 days post-irradiation (or sham-irradiated) kidney tissue. DNA (cytosine-5-)-methyltransferase 1 (DNMT1; panel A1,2,3), DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A; pan B1,2,3), DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B, panel C1,2,3). Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc analysis. (*; significant data compared to control, ▲; significant data compared to 5 Gy radiation group).

3.3 Discussion

3.3.1 Kidney Renin-Angiotensin System

Mechanism of radiation-induced tissue damage is not clear, however, studies showed the efficacy of ACEIs (angiotensin converting enzyme inhibitors) in preventing or alleviating radiation-induced tissue injury in organs such as kidney, lung (46) and brain (47).

This study did not show any change caused by 5 Gy radiation in angiotensinogen, ACE, AgtR1A and SP1 expression levels. There is no evidence of radiation-induced activation or upregulation of systemic or intrarenal RAS (26). However, the efficacy of ACEIs and AT1RA in preventing or reducing radiation-induced injury suggests an ongoing interaction between radiation and Ang II (48). It is suggested that even RAS, the normal function may be deleterious in an irradiated kidney (26). Ionizing radiation and RAS similarly induce their effects via ROS production. Collins et al. 2008 showed ionizing radiation activates NADPH oxidase which significantly contribute to radiation-induced ROS production in cells (49). Our data did not show radiation-induced activation of local renal RAS.

MDS-5Gy-MDS and 5 Gy-MDS group demonstrated significantly lower expression levels of angiotensinogen at all three time points. Sporadic lower expression levels of ACE, renin, AgtR1A and SP1 were also observed. To understand the reason behind these observations, we must examine the biological regulation and function of angiotensinogen.

In this study, it is shown that kidney tissue from the MDS-5Gy-MDS is protected from radiation-induced upregulation of eNOS. Possibly lower expression level of eNOS leads to less $ONOO^-$ production. In the 5 Gy group overexpression of eNOS could over activate the cycle between AT1R, NADPH oxidase and mitochondria (Figure 8). In the MDS-5 Gy-MDS group eNOS was not elevated and by potentially lesser ROS feedback to NADPH oxidase

enzyme, AT1R is also less activated. Considering the positive feedback control of AT1R activation on the expression level of angiotensinogen, the relatively lower signal causes reduced expression levels of angiotensinogen in MDS-5GY-MDS and 5 Gy-MDS compared to the control.

Decreased angiotensinogen expression levels in MDS-5 Gy-MDS group (Figure 3A), suggested that RAS system compensates the reduced signaling by elevated levels of ACE and renin since in the face of elevated levels of Ang II, ACE and renin are downregulated (50). However, ACE and renin expression levels were initially decreased, probably because although angiotensinogen expression level is significantly lower than control, radiation-induced oxidative stress is sufficient to maintain the cellular ROS level high enough to mimic a normal level of AngII and activation of NADPH oxidase.

At 30 days post-radiation, decreased angiotensinogen expression levels in 5Gy-MDS group is accompanied by elevated ACE expression level (potentially increase the production of AngII) and reduction in Ang II receptor. AGTR1A expression level is controlled by SP1 transcription factor (51) and both genes showed down-regulation.

Lower expression levels of angiotensinogen in MDS-5 Gy-MDS and 5 Gy-MDS group suggests a lower activity level of RAS and production of reactive oxygen species via function of NADPH-oxidase enzyme. Considering Ang II cellular signaling and role of NADPH oxidase, It is speculated that radiation-induced ROS production is lower in MDS-5 Gy-MDS and 5 Gy-MDS compared to 5 Gy group. Collectively data suggest MDS has the potential to protect the kidney from radiation-induced tissue damage. According to RAS-related genes expression levels throughout the experiment, MDS can protect the tissue not just from initial irradiation induced tissue damage but also from radiation-induced long term oxidative stress.

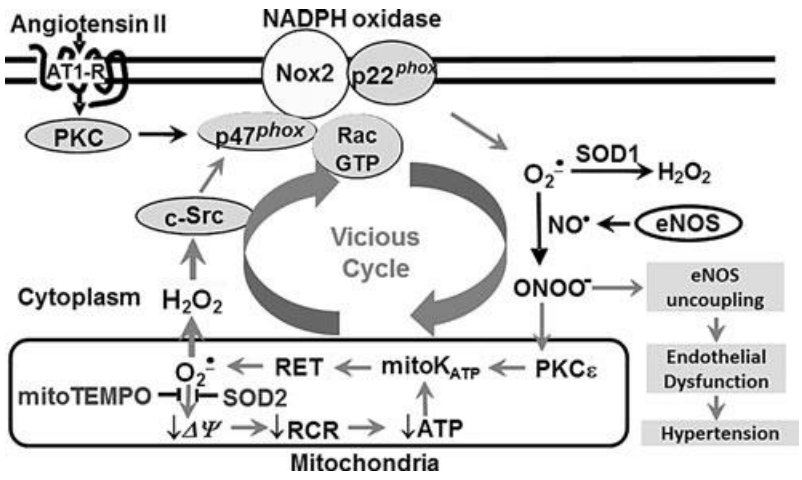

Figure 8 Signaling network between AngII, NADPH oxidase and mitochondria (59)

3.3.2 Cellular Antioxidant System

In the present study, NRF2 transcription levels were raised in response to 5 Gy whole body radiation in the absence or presence of MDS (2 and 30 days post-radiation). NRF2 gene expression in cells is constitutive and protein has a short half-life of 15 minutes. In response to oxidative damage, NRF2 becomes activated via its release from Keap1, which causes constitutive NRF2 degradation in the proteasome. After release from Keap1, NRF2 translocates from the cytoplasm to nucleus (52). This study showed a significant induction at the mRNA expression levels of NRF2 due to radiation-induced oxidative stress in kidney tissue which was reported previously (53).

Hydrogen peroxide is one the main reactive oxygen species produced by radiation in cells. Hydrogen peroxide is produced as a result of water radiolysis and catalysis of superoxide by SODs. In cells, hydroxyl radicals generated from hydrogen peroxide causes damage to DNA, proteins and lipids. GPX1 and catalase are two main enzymes responsible for detoxification of hydrogen peroxide. Catalase has a high reaction and turnover rate which means it is very valuable in detoxification of higher concentrations of hydrogen peroxide.

Cells depend on catalase activity to eliminate oxidative stress. The reaction of catalase and hydrogen peroxide converts active catalase to inactive enzyme forms. Catalase expression is elevated due to oxidative stress and for the system to sustain the activity of catalase, GPX1 levels are increased as well (54). In 5 Gy group, GPX1-catalase cooperativity provides the cell with H_2O_2 protection. On the other hand, in MDS-5 Gy-MDS group, MDS provided protection against radiation-induced ROS so although catalase expression levels were elevated there was no elevation in expression levels of GPX1.

Radiation-induced activation of DNA damage sensors initiates signal transduction pathway that activates calcium-dependent NOS. In this study expression level of eNOS was elevated in response to 5 Gy radiation although the enzymes are reported to be constitutively expressed and not easily induced by stimuli (55). Mitochondria is the main source for production of reactive oxygen species in cells and is the main target for NO. Cytoplasmic localized eNOS overexpression contributes to the production of $ONOO^-$ (peroxynitrite) since NO diffusion is very rapid in the cytoplasm (56). Radiation-induced superoxide anion production causes overactivation of eNOS consequently increases NOS uncoupling which elevates production of $ONOO^-$ and causes cytoplasmic damage. MDS-5 Gy-MDS group on the other hand did not show any increase in NOS3 expression levels. Collectively, besides the reported MDS potential to reduce nutritive stress in mitochondria (57) we showed it can protect the kidney tissue from radiation-induced elevated eNOS expression levels in the cytoplasm.

Elevated eNOS expression levels in the irradiated group may cause higher production levels of NO. NO radical has high affinity to react with superoxide free radical and production of $ONOO^-$.This free radical is highly active and damages DNA, causes protein nitration and

deactivation and also activates NF-κB (55). Also $ONOO^-$ causes an overproduction of superoxide anion in mitochondria and consequence elevation in production cytoplasmic hydrogen peroxide. Another important aspect of increased $ONOO^-$levels in 5 Gy radiated group is deactivation of SOD2, which is a vital part of the cellular defense against oxidative stress in mitochondria (58). We speculate that MDS reduced the radiation-induced ROS and therefore no significant change was seen in NOS3 expression levels compared to the control group. This may well preserve the functionality of SOD2 enzyme compared to the recipients of 5 Gy whole body radiation and disrupt the cellular circle which leads to overproduction of ROS in cells.

In 5 Gy group at 2 days post-radiation radiation-induced superoxide is catalyzed by SOD1 or reacts with NO. It is suggested that by 30 days post-radiation, there is a higher concentration of ROS due to the effects of initial radiation-induced ROS on mitochondria and NDAPH oxidase (part of Ang II signaling pathway) which activates a circle of events between NADPH oxidase and mitochondria which eventually produces a high level of ROS. In this condition greater portion of superoxide is engaged in eNOS uncoupling and less is catalyzed with SOD1 which all together led to downregulation of SOD1 gene. It is suggested that SOD1 expression levels are lower in the MDS-5 Gy-MDS group due to lower cytoplasmic superoxide.

SOD2 or mitochondrial SOD regulates the redox homeostasis in mitochondria by converting mitochondrial superoxide to hydrogen peroxide. Overexpression of SOD2 in mitochondrial oxidative stress, protects mitochondria's function, membrane potential and ATP production (59). Radiation oxidative stress affects mitochondria in the long term by decreasing the activity of mitochondrial complex I and III. (60). SOD2 expression level was

elevated in 5 Gy group by 30 days. This finding could be explained as follow. In the 5 Gy group as a result of positive feedback between NADPH oxidase and mitochondria, ROS levels increased by time and caused an imbalance in mitochondrial oxidative state 30 days post-radiation. As a consequence of this possible higher oxidative stress, the expression level of SOD2 was elevated in 5 Gy group. However, the expression level of SOD2 did not change in MDS-5 Gy-MDS group which might indicate that MDS protected kidney tissue from radiation-induced ROS formation in mitochondria.

MDS increases the activity of mitochondrial complex III and IV and as a result, causes increased ATP production, preservation of mitochondrial function and at the same time reduces the free radical production related to complex III in aging mice (61). In MDS treated animals (MDS-5 Gy-MDS) SOD2 gene expression was downregulated suggesting MDS effects on decreasing the need for cells to induce SOD2 expression levels to neutralize the radiation-induced ROS in mitochondria.

3.3.3 DNA Damage Repair and Cell Cycle Progression

Study of kidney tissue after exposure to ionizing radiation showed an evidence of cell cycle arrest as it was indicated by elevated expression levels of CDKN1A in 5 Gy and MDS-5Gy-MDS group. This observation was fully known as CDKN1A is inducible by a variety of stimuli like ionizing radiation. It was suggested that in irradiated kidney tissue (5 Gy) mitochondrial oxidative stress increases by time (SOD2 gene upregulation-30 days post-radiation). Increased oxidative stress in mitochondria increases the hydrogen peroxide levels in the cytoplasm and ultimately causes DNA damage in the nucleus. Hydrogen peroxide causes DNA strand break (62) and base damage (63). Upregulated GADD45A suggests

higher DNA damage compared to the control 30 days post-radiation. This hypothesis is further supported by an elevated PER2 expression level in 5 Gy group, as PER2 is engaged in the regulation of nucleotide excision repair which is needed for all base lesion repair (64). In conclusion, 5 Gy group demonstrated evidence of radiation-induced DNA damage.

On the other hand, it was suggested that MDS protects the mitochondria from radiation-induced chronic oxidative damage and DNA damage by evidence from expression levels of PER2 and GADD45A which did not indicate any outstanding DNA damage compared to the control.

H2AX phosphorylation happens in response to DNA double-strand breaks (DSBs). The phosphorylated form or γH2AX is important in initiation and assembly of DNA repair proteins at the site of damage and activation of checkpoint proteins. In this study, we measured the transcription levels of H2AX and we did not study the phosphorylated form.

Radiation-induced DNA damage repair is completed within hours. However, expression levels of H2AX in 5 Gy group is persistently high at 2 and 30 days post-radiation. This evidence could reflect the response to DNA damage (65). Previous studies showed MDS's potential in the reduction of basic and induced DNA damage (42). In the present study, MDS group H2AX expression level was interestingly increased compared to the control even though no external source of DNA damage was introduced. Having this in mind, MDS-5 GY-MDS group showed no change in H2AX expression level 2 days post-radiation. This observation could further be investigated to show a connection between H2AX expression levels and MDS function in the cell.

3.3.4 Physiological Stress Response

Utilization of glucocorticoids at cellular level depends on the function of HSD11B1 (converts the cortisol to active cortisone) and glucocorticoid receptor (GR). HSD11B1 expression levels are affected by a variety of factors including glucocorticoids levels which increases HSD11B1 expression. Cortisol serum levels increase significantly 4-6 hours after the stress and utilization of cortisol happen in correlation with cortisol's release time. Data from HSD11B1 (significant decrease compared to the control) and GR (no change compared to the control) expression levels 2 days post-irradiation is uninformative and cannot be interpreted as radiation-induced cellular physiological stress. But the data is explainable as follow. Stress causes release of glucocorticoid from the adrenal gland. Adrenal gland has a low hormone level afterward that will be replenished within few days post-stress by activation of hormone synthesis. 2 days post-radiation HSD11B1 expression level is low in the kidney tissue since systemic glucocorticoid level is not back to the normal levels. This suggestion is supported by considering the activation of catecholamine biosynthesis pathway in adrenal by 2 days post-radiation (chapter 4.2.3) which suggest a prior higher levels of glucocorticoid release from adrenal gland.

On the other hand, MDS-5Gy-MDS appear to further decrease the HSD11B1 expression levels accompanied by lower cellular expression levels of GR 30 days post-irradiation. Considering lack of activation of catecholamine biosynthesis pathway in MDS-5 Gy-MDS post radiation (chapter 4.2.3) lower levels of HSD11B1 is due to the protection of MDS against radiation-induced stress and release of glucocorticoid from the adrenal. GR

expression level was decreased compared to control even though higher GR level was expected due to the lower glucocorticoid level (66).

3.3.5 DNA Methyltransferase Family

Radiation is a genotoxic agent that affects the genome integrity and instability. DNA global methylation was specifically interesting due to its possible long-term effects such as carcinogenesis. Presentation of DNA hypomethylation in a tissue depends on a variety of specific factors such as radiation dose and course of delivery (acute delivery causes hypomethylation), sex and organ (radiation induced hypomethylation in liver, lack of it in the brain) (67). Sex is important as female animals are more prone to radiation-induced hypomethylation (68). Radiation-induced DNA hypomethylation is also believed to be related to DNA damage and DNA repair (68). DNA methyltransferase activity was indirectly studied via expression levels of important maintenance (DNMT1) and *de novo* DNA methyltransferases (DNMT3A&3B). Among DNMTs genes 5 Gy radiation caused an aberration in the normal pattern of DNMT3B expression (30 days post-radiation). DNMT3B downregulation could lead to DNA hypomethylation. MDS-5 Gy-MDS and 5 Gy-MDS tissue were protected from radiation-induced decreased DNMT3B expression level and the expression levels of the enzyme remained unchanged compared to the control. Higher expression levels of DNMT1 was observed in the MDS group at 30 and 120 days post-radiation which could be explained since DNMT1 is recognized to be responsible for DNA methylation after replication and a higher level of DNA synthesis in cells could be accompanied by higher levels of DNMT1 (69).

4.0 Adrenal

4.1.1 Introduction

According to MDS's function as antioxidant and free radical scavenger, mitochondrial support and DNA protector we hypothesized that MDS protects the adrenal tissue from the radiation-induced production of free radicals and DNA damage. We also hypothesized that MDS could mitigate the radiation-induced systemic stress and activation of HPA axis.

4.1.2 Objectives

To assess adrenal tissue for:

A) Radiation-induced changes in expression level of genes involved in the cellular antioxidant system, DNA repair, cell cycle progression, DNA methyltransferase enzymes and markers of physiological stress and HPA axis.

B) Expression level of the above genes in groups receiving MDS in combination with radiation.

4.2 Results

Results from the genes studied in adrenal tissue including cellular antioxidant system, DNA damage repair, cell cycle progression, catecholamine biosynthesis pathway, markers of cellular physiological stress response and DNA methyltransferase family, are summarized in Table 5.

		mRNA Expression Changes Compared to Control											
		2 Day				30 Day				120 Day			
	Gene	MDS	5 Gy	MDS 5Gy MDS	5 Gy MDS	MDS	5 Gy	MDS 5Gy MDS	5 Gy MDS	MDS	5 Gy	MDS 5Gy MDS	5 Gy MDS
Cellular anti-oxidant system	NRF2 - nuclear factor 2	↔	↑	↑									
	Catalase	↔	↑	↑									
	GPX1-glutathione peroxidase 1	↔	↑	↔									
	NOS3 - endothelial nitric oxide synthase	↔	↔	↔									
	SOD1 – superoxide dismutase 1	↔	↔	↔									
	SOD2 – superoxide dismutase 2	↔	↔	↓									
	SOD3 – superoxide dismutase 3	↔	↔	↔									
DNA damage repair/ cell cycle progression	CDKN1A – cyclin-dependent kinase inhibitor 1	↔	↑	↑									
	GADD45 – Growth arrest and DNA-damage-inducible protein	↔	↔	↔									
	H2AX – H2A histone family, member X	↔	↔	↔									
	PER2 –period circadian clock 2	↔	↑	↑									
HPA axis	PNMT – phenylethanolamine-N methyltransferase	↔	↑	↔	↔	↔	↔	↓	↔	↔	↔	↓	↔
	DBH – dopamine beta hydroxylase	↔	↔	↔	↔	↔	↔	↓	↔	↓	↓	↓	↔
	TH – tyrosine hydroxylase	↔	↔	↔	↔	↔	↔	↓	↔	↔	↔	↓	↔
	EGR1 – early growth response 1	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
	Sp1 – trans-acting transcription factor	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
	AP2 – transcription factor AP-2, alpha	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
Physiological stress	HSD11β1 – 11β-hydroxysteroid dehydrogenase type 1	↔	↔	↔									
	Nr3c1 – glucocorticoid receptor	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
DNA methyl-transferase	DNMT1-DNA methyltransferases	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
	DNMT3A-DNA methyltransferases	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	↔
	DNMT3B-DNA methyltransferases	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔

Table 5 Adrenal mRNA expression Analysis Summary. mRNA Expression Changes Compared to Control (Group#1), ↔=no change; ↓=decrease; ↑=increase

4.2.1 Adrenal Cellular Antioxidant System

5 Gy radiation-induced changes, in the cellular antioxidant system in adrenal tissue, was analyzed by measuring mRNA levels of the main antioxidant genes. mRNA expression analysis demonstrated a 2.3-fold increase in NRF2 mRNA levels in 5 Gy group compared to the control 2 days post-radiation. NRF2 expression level was increased by 2.2-fold compared

to the control in MDS-5 Gy-MDS group and MDS did not modify radiation-induced NRF2 upregulation (Figure 9A).

Study of mRNA expression levels of catalase in adrenal tissue showed a 2.4-fold increase in 5 Gy group compared to the control group 2 days post-radiation. Catalase expression level in MDS-5 Gy-MDS was increased by 1.6-fold compared to the control (Figure 9B).

Study of GPX1 expression levels in adrenal tissue showed a 2.13-fold increase compared to the control in 5 Gy group. In MDS-5 Gy-MDS group MDS protected the kidney tissue from radiation-induced oxidative stress in a way that GPX1 (Figure 9C) expression levels did not change compared to the control. Study of NOS3 (Figure 9D) and SOD1 (Figure 9E) expression levels in adrenal tissue, 2 days post-irradiation showed no significant change compared to the control group due to 5 Gy whole body radiation. In MDS-5 Gy-MDS group MDS did not modify the adrenal response to radiation.

5 Gy whole body radiation caused no significant change in expression levels of SOD2 compared to the control 2 days post-radiation. However, in MDS-5 Gy-MDS group SOD2 mRNA levels decreased to 3-fold of the control group (Figure 9F).

Taken together mRNA expression levels of antioxidant genes, suggest MDS administration in the MDS-5 Gy-MDS group protected adrenal tissue from 5 Gy whole body radiation-induced oxidative stress in cytoplasm and mitochondria.

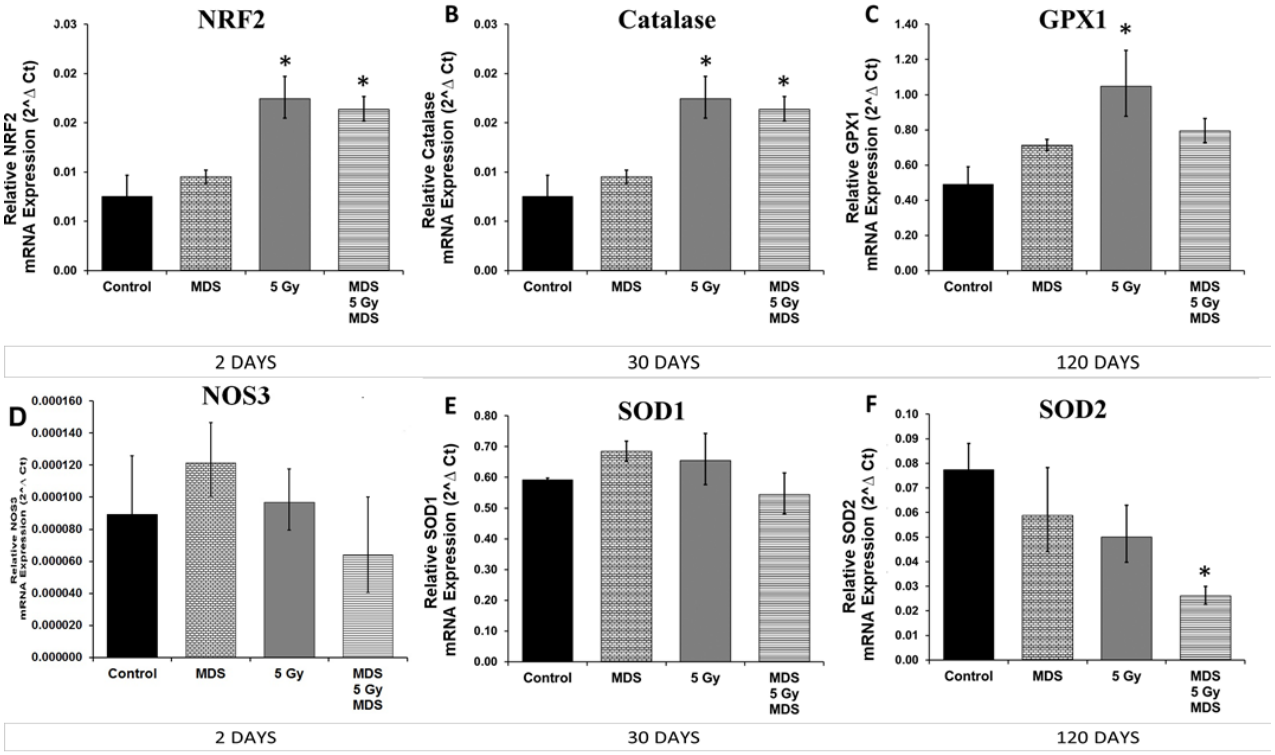

Figure 9 RT-qPCR mRNA expression analysis of markers of endogenous antioxidants system in adrenal 2 days post-irradiation (or sham-irradiated) adrenal tissue. mRNA expression of nuclear factor-like 2 (NRF2; panel A), catalase (panel B), glutathione peroxidase 1 (GPX1; panel C), nitric oxide synthase 3 (NOS3; panel D), superoxide dismutase 1 (SOD1; panel E), superoxide dismutase 2 (SOD2; panel F) were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc analysis. (*; significant data compared to control).

4.2.2 DNA Damage and Cell Cycle Progression

RT-qPCR analysis of the adrenal gland 2 days post-radiation demonstrated that 5 Gy whole body radiation resulted in a 10.7-fold elevation in CDKN1A mRNA levels compared to the control. Similarly, the expression level of CDKN1A in MDS-5 Gy-MDS group showed a 10.7-fold increase compared to the control (Figure 10A). The robust increase in cdkn1a mRNA levels in both groups suggest induction of cell cycle arrest after radiation exposure.

mRNA expression analysis of GADD45 and H2AX 2 days post-radiation demonstrated no significant change in the expression levels compared to the control caused by 5 Gy whole body irradiation. MDS administration as in MDS-5 Gy-MDS did not modify GADD45 and H2AX expression levels compared to the control in adrenal tissue (Figure 10B&C).

mRNA expression analysis of PER2 in the adrenal tissue 2 days post-radiation showed a 2.6-fold increase in PER2 expression levels in 5 Gy group compared to the control. MDS-5 Gy-MDS group also showed the same trend and PER2 expression levels raised to 2.7-fold compared to the control (Figure 10D).

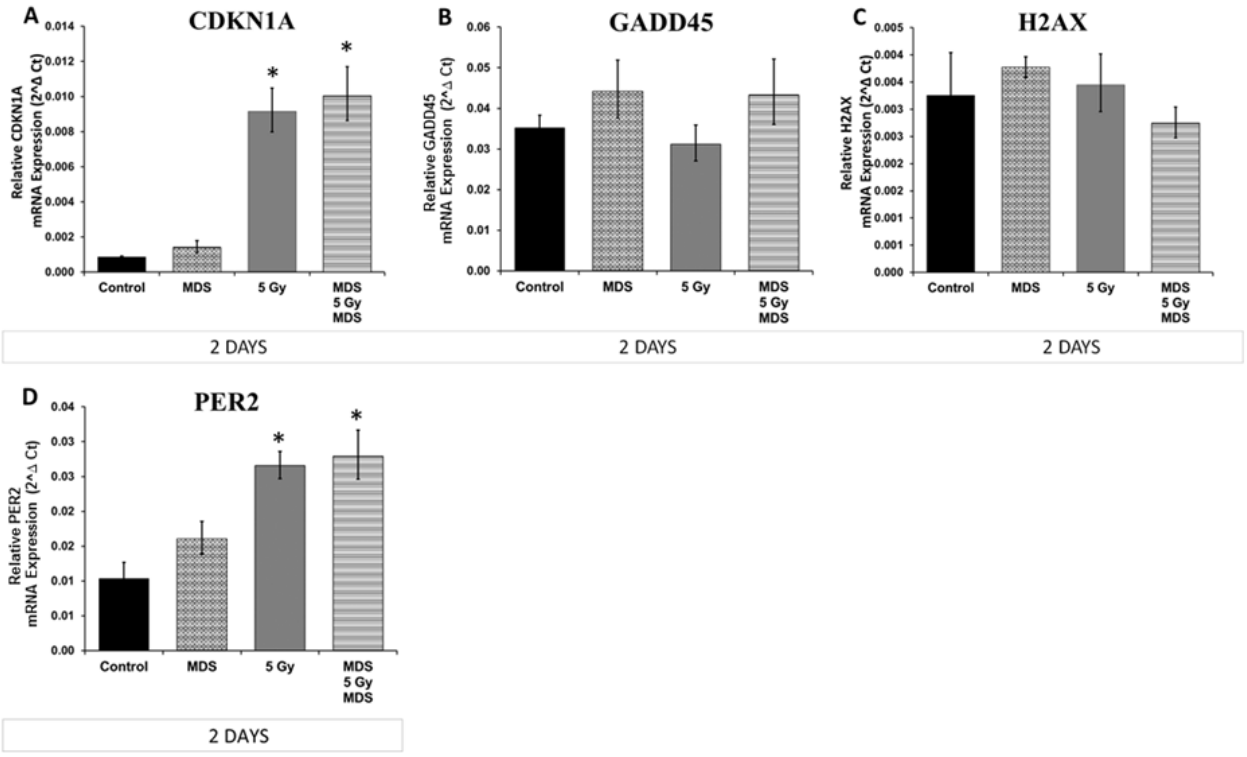

Figure 10 RT-qPCR mRNA expression analysis of markers of cell cycle regulation 2 days post-radiation (or sham-irradiation) in adrenal tissue. mRNA expression of cyclin-dependent kinase inhibitor 1 (CDKN1A; panel A), Growth arrest and DNA-damage-inducible protein (GADD45; panel B), H2A histone family (H2AX; panel C), period circadian protein 2 (PER2; panel D) were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc analysis. (*; significant data compared to control).

4.2.3 Catecholamine Biosynthesis Pathway

Irradiation-induced changes in catecholamine biosynthesis in the adrenal gland was assessed by measuring the transcription levels of genes involved in catecholamine biosynthesis pathway. Phenylethanolamine-N-methyltransferase (PNMT) catalyzes the conversion of norepinephrine to epinephrine. mRNA expression analysis of PNMT in the adrenal tissue showed a 1.7-fold increase compared to the control group due to 5 Gy whole body radiation at 2 days post-radiation. At 30 and 120 days post-radiation PNMT expression levels were not significantly different from the control. mRNA expression level analysis also showed the ability of MDS to modify the radiation-induced increase in PNMT expression levels as it is demonstrated by significantly lower expression levels of PNMT in MDS-5 Gy-MDS and 5 Gy-MDS groups compared to the 5 Gy radiation at 2 days post-radiation. Although PNMT expression level in MDS-5 Gy-MDS group was not significantly different from the control group 2 days post-radiation, it was significantly decreased by 1.8 and 2.1-fold compared to the control group 30 and 120 days post-radiation. Early increase in PNMT expression level in 5 Gy whole body radiation showed activation of catecholamine biosynthesis due to radiation induced stress (Figure 11A). PNMT expression levels in MDS-5 Gy-MDS and 5Gy-MDS groups, on the other hand, demonstrated MDS potential to protect the body from radiation-induced physiological stress and need to synthesize and secrete epinephrine.

Dopamine beta hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine in the catecholamine biosynthesis pathway. RT-qPCR analysis of the adrenal gland demonstrated that 5 Gy whole body irradiation did not result in any significant change

in the cellular expression level of DBH at 2, 30 days post-radiation. However, 120 days post-irradiation DBH mRNA level decreased by 1.4-fold compared to the control in the adrenal tissue. Study of DBH expression levels showed that the MDS-5Gy-MDS demonstrated a significant reduction in DBH expression levels by 1.5 and 2.5-fold compared to the control group 30 and 120 days post-radiation. MDS group also showed a significantly lower DBH expression level compared to the control at 120 days post-radiation (Figure 11B). MDS-5 Gy-MDS group showed to have lower DBH expression level potentially due to lower stress after 5 Gy whole body radiation and lower catecholamine release.

Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to L-dopa, 5 Gy whole body radiation did not result in any significant changes in enzymes expression levels at any of 2, 30 or 120 days post-radiation. Although MDS-5 Gy-MDS group did not show any significant changes in TH expression levels compared to the control group 2 days post-radiation, enzyme expression levels were significantly decreased to 1.5 and 2.5-fold compared to the control at 30 and 120 days post-radiation (Figure 11C).

Important transcription factors in regulating gene expression in catecholamine biosynthesis pathway including, early growth response 1 (EGR1) and trans-acting transcription factor (Sp1), AP-2 alpha and glucocorticoid receptor (GR) were studied to determine the effect of 5 Gy whole body radiation and MDS on the activity of catecholamine biosynthesis pathway. RT-qPCR analysis of adrenal tissue demonstrated no change in GR expression levels due to 5 Gy whole body radiation in either of the time point. Although no significant changes in GR expression levels were seen in MDS-5 Gy-MDS group, GR

expression levels were increased significantly in 5 Gy-MDS group compared to the control 120 days post-radiation (Figure 11D).

Study of the effect of 5 Gy whole body radiation on SP1, AP2 and Egr-1 mRNA levels revealed no significant changes due to radiation at any of the 2, 30 and 120 days post-radiation. Moreover, MDS administration did not change the expression levels of these transcription factors in any of the MDS, MDS-5 Gy-MDS and 5 Gy-MDS groups (Figure 11E, F&G). In general, MDS protected the body from radiation-induced stress and activation of HPA axis as it was demonstrated by the lack of activation of catecholamine biosynthesis pathway.

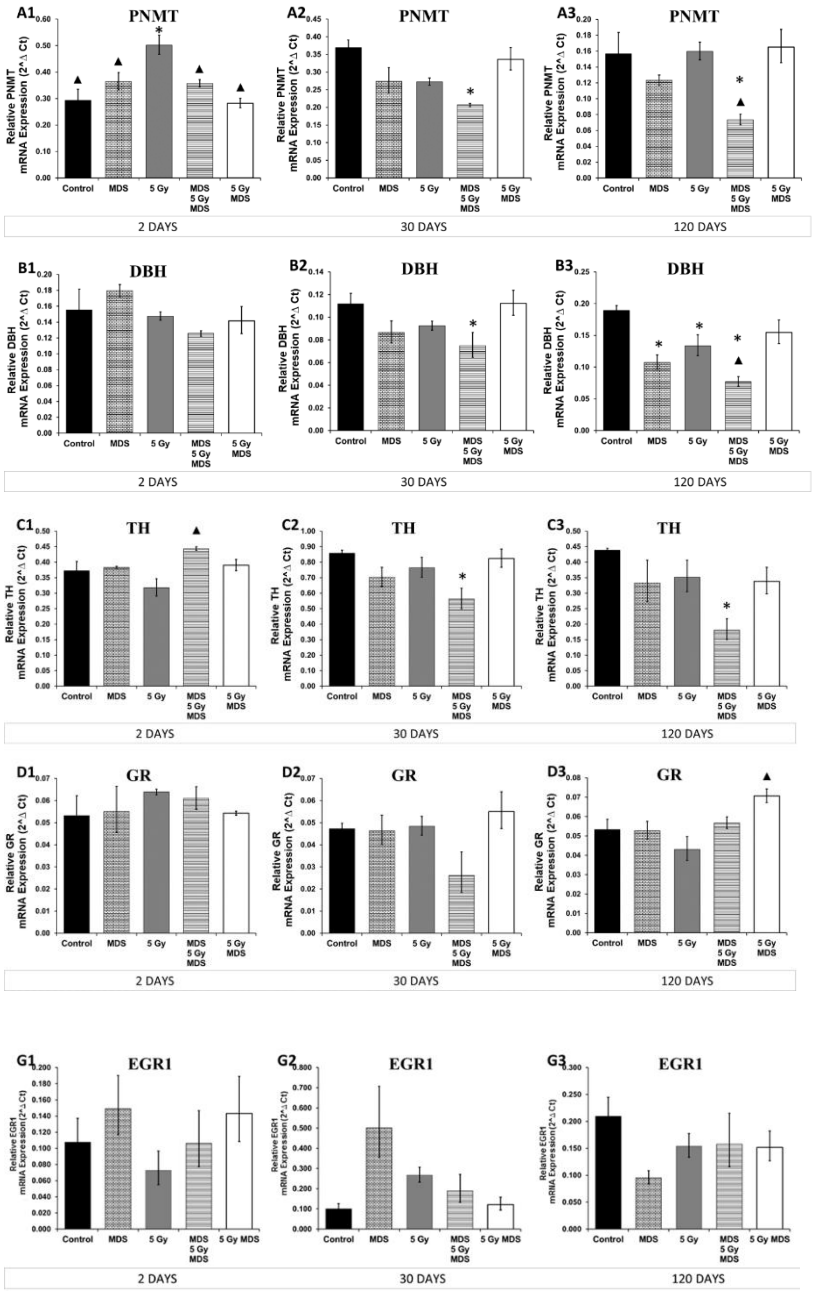

Figure 11 RT-qPCR mRNA expression analysis of markers of catecholamine synthesis in 2 days post-irradiation (or sham-irradiated) in adrenal. mRNA expression of phenylethanolamine-N-methyltransferase (PNMT; panel A), dopamine beta hydroxylase (DBH; panel B), tyrosine hydroxylase (TH; panel C), glucocorticoid receptor (GR; panel D), trans-acting transcription factor (Sp1; panel E), transcription factor AP-2, alpha (AP2; panel F), early growth response 1 (Egr-1; panel G), were determined relative to β-actin and ribosomal protein L29 reference genes were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-

hoc analysis. (*; significant data compared to control, ▲; significant data compared to 5 Gy radiation group).

4.2.4 Physiological Stress Response

RT-qPCR analysis of markers of physiological stress response in adrenal gland demonstrated 5 Gy whole body radiation had no effect on the expression level of HSD11B1 at 2 days post-radiation. On the other hand, MDS-5 Gy-MDS showed a significant 2.5-fold decrease in cellular expression levels of HSD11B1 compared to the control group (Figure 12A).

Study of GR mRNA expression levels in adrenal gland did not demonstrate any significant changes due to 5 Gy whole body radiation two days post-radiation. Moreover, MDS-5 Gy-MDS group did not change the cellular GR expression levels compared to the control group (Figure 12B).

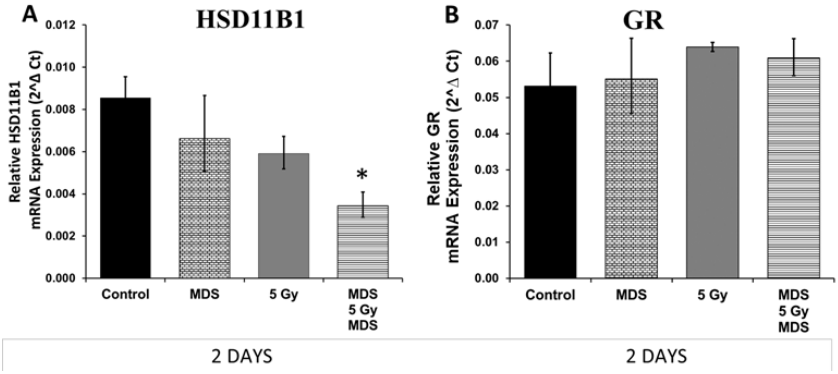

Figure 12 RT-qPCR mRNA expression analysis of markers of physiological stress response in 2 days post-irradiation (or sham-irradiated) adrenal tissue. mRNA expression of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1; panel A), nuclear receptor subfamily 3, , group C, member 1 or Glucocorticoid receptor (NR3C1; panel B) were determined relative to β-actin and ribosomal protein L29 reference genes. Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc analysis. (*; significant data compared to control).

4.2.5 DNA Methyltransferase Family

mRNA expression levels of DNA methyltransferase enzymes which control the DNA methylation in adrenal tissue has shown that 5 Gy whole body radiation did not cause any significant change in the expression level of DNMT1 enzyme compared to control group. MDS-5Gy-MDS and 5 Gy-MDS group also did not demonstrate any significant changes in DNMT1 expression levels compared to the control group (Figure 13A).

Study of DNMT3A expression levels in adrenal tissue showed that 5 Gy whole body radiation did not cause any changes in enzymes expression levels. However, expression level of DNMT3A in MDS-5 Gy-MDS group showed a significant decrease by 2-fold compared to the control group 120 days post-radiation (Figure 13B).

Study of DNMT3B expression levels in adrenal tissue did not demonstrate any changes due to 5 Gy whole body radiation. MDS did not cause any change in enzyme's expression level in any of the time points (Figure 13C).

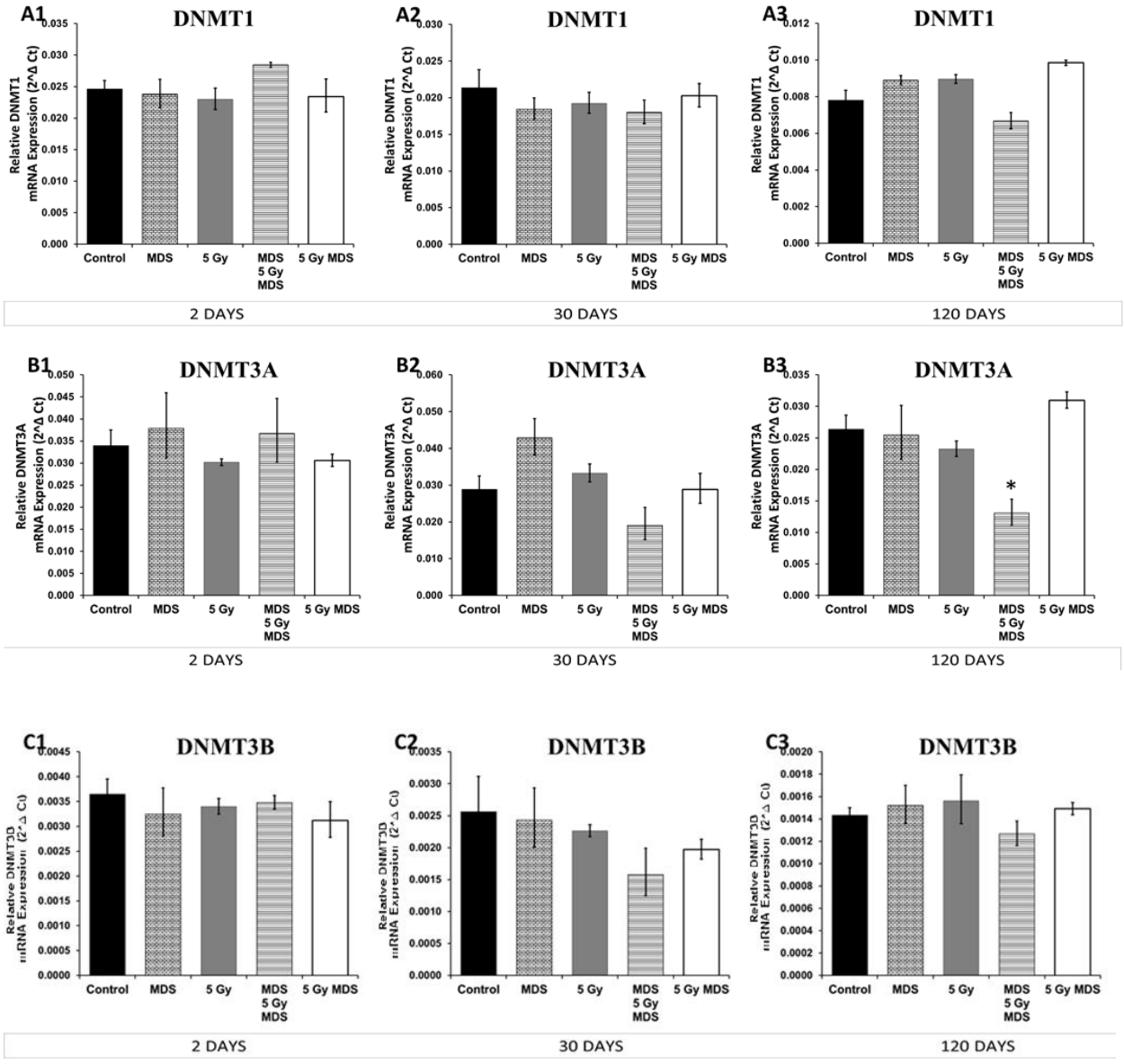

Figure 13 RT-qPCR mRNA expression analysis of markers of DNA methylation in 2,30 and 120 days post-irradiation (or sham-irradiation) adrenal tissue. DNA (cytosine-5-)-methyltransferase 1 (DNMT1; panel A1,2,3), DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A; panel B1,2,3), DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B, panel C1,2,3). Each group consisted of 5 animals. Statistical significance was determined at $p < 0.05$ using one-way ANOVA followed by Tukey's post-hoc analysis. (*; significant data compared to control).

4.3 Discussion

4.3.1 Cellular Antioxidant System

NRF2 expression levels were induced by 5 Gy radiation in the 5 Gy and MDS-5 Gy-MDS groups. Radiation oxidative stress in cytoplasm causes elevated catalase levels accompanied by GPX1 upregulation which protects the catalase from being damaged and deactivated by hydrogen peroxide. The 5 Gy group demonstrated significantly higher catalase and GPX1 levels which suggest higher levels of superoxide in the tissue. Presuming in the MDS-5Gy-MDS group, MDS reduces the production of superoxide by NADPH oxidase and hydrogen peroxide from mitochondria. If true in this group catalase expression seems to be directly controlled with upregulation in NRF2 but due to the lower possible cytoplasmic oxidative stress GPX1 expression levels did not change.

The cellular antioxidant defense has tissue-specific properties. Adrenal tissue shows highly specific features including a very high level of non-enzymatic antioxidants like vitamin C, E and glutathione. Antioxidant enzymes like SOD1, SOD2 and GPX are very active in the adrenal. However, catalase has a very low activity level. Expression levels of these enzymes correspond well with their activity levels as SOD1,2 and GPX1 mRNA is abundant but catalase mRNA levels are very low (70). In this study, it was suggested that higher expression levels and activity of antioxidant enzymes and also the considerable non-enzymatic antioxidant capacity of adrenal tissue seems to relatively protect the tissue from radiation-induced oxidative stress in short-term (2 days) as it is evidenced by the lack of change in the expression levels of NOS3 and SOD2.

MDS may further protect the adrenal tissue from radiation-induced ROS production considering evidences such as lack of radiation-induced GPX1 upregulation and also

downregulation of SOD2 at 2 days post-radiation which reflects lower mitochondrial oxidative stress.

4.3.2 DNA Damage Repair and Cell Cycle Progression

The study showed a strong indicator of cell cycle arrest in 5 Gy and MDS-5Gy-MDS group in adrenal tissue 2 days post-radiation as it was demonstrated by elevated expression levels of CDKN1A. Activation of DNA repair mechanism which was predicted by elevated PER2 expression level was also activated in both MDS-5 Gy-MDS and 5 Gy groups. In adrenal tissue, the MDS-5 Gy-MDS data suggest that MDS did not provide any benefit to protect the DNA in adrenal tissue from radiation-induced DNA damage at 2 days post-radiation.

4.3.3 Catecholamine Biosynthesis Pathway

Stress causes activation of HPA axis. ACTH (Adrenocorticotropic hormone) is released from the pituitary gland and causes synthesis and release of glucocorticoids from the adrenal cortex. Glucocorticoids then directly affect adrenal medulla and in return, Epinephrine is synthesized and released. Its worthy to mention stressor's type, and duration of stress influence the transcriptional changes which affect the catecholamine biosynthesis (71). Unfortunately, no similar study was available that evaluated the radiation as a stressor.

Epinephrine and glucocorticoid synthesize compensate for the hormone released due to the stress. PNMT regulation is specifically responsive to hormonal regulation by glucocorticoids as corticosteroids binds to corticoid receptors and activates PNMT promoter (34). Elevation in PNMT expression levels in 5 Gy whole body radiation was sustained for 2

days post-radiation. Although prolonged increase in PNMT expression levels has been reported in stress with longer periods (immobilization for 2 hours) (71), in acute stress on the other hand, PNMT (peaked 6-8 hours) is reported to be transient and resolve and restored to normal levels within 24 hours (72). Evaluation of TH and DBH expression levels in 5 Gy group showed no similar elevation which could be due to importance of the neural input in their expression level (73) and their faster response to stress. Rise in the TH and DBH levels could be detected at earlier time (less than 24 hours) (74). More long-term effect of radiation on catecholamine biosynthesis pathway appeared as DBH downregulation in the 5Gy group, 120 days post-radiation. The expression levels of transcription factors such as EGR-1, SP1, AP2 and GR 2 days post-radiation showed no significant changes. Data could be explained considering transcription factors fast response to the stress (within hours post-stress) (72) which means a change in the expression level of transcription factors is detectable at an earlier time points. Collectively data suggest radiation induced stress in the 5 Gy group which was demostrated by significant increase in expression levels of PNMT.

Data suggests that MDS protected irradiated animals from radiation-induced stress and activation of HPA axis. The protection was achieved by both MDS-5Gy-MDS and 5G-MDS groups as PNMT expression levels were not induced 2 days post-radiation. MDS-5Gy-MDS group also demonstrated significantly lower expression levels of PNMT, DBH and TH compared to the control 30 and 120 days post-radiation. This finding is explainable regarding this idea that AngII induces the catecholamine release (75) and biosynthesis (76). Lower expression levels of angiotensinogen (as in MDS-5Gy-MDS group) is followed by lower synthesis and release of catecholamine from adrenal. Although the angiotensinogen expression level was also lower in the 5 Gy-MDS group, the PNMT, DBH and TH expression

levels were not affected at 30 and 120 days post-radiation. These changes in enzymes expression levels were not accompanied by changes in the expression levels of SP1, AP2 and EGR1. This finding could be justifiable by the considering a lag time between the increase in expression level of the transcription factor and the target gene or by the fact that post-translational activation of the transcription factor happened without any changes in expression level.

Collectively, data from mRNA expression levels suggest MDS has the potential to protect the mice from radiation-induced physiological stress and as a result demonstrated lower HPA axis activity.

MDS demonstrated an ability to protect the kidney tissue from radiation-induced stress and could be used as prophylaxis (MDS-5Gy-MDS) or treatment (5Gy-MDS). However, the consequence of these diets are varied through the time. In the MDS-5 Gy-MDS group, catecholamine biosynthesis is predictably lower as it is suggested by lower expression levels of PNMT, DBH and TH at 30 and 120 days post-radiation.

4.3.4 Physiological Stress Response

Regarding the fact that cortisol is released shortly after the stress, mRNA expression levels of HSD11B1 and GR should have been studied at a much shorter time to reflect the glucocorticoid release due to the radiation-induced stress.

Considering the positive effect of glucocorticoids on the regulation of HSD11B1 (77), Data in the adrenal tissue suggests the presence of normal levels of glucocorticoid 2 days after radiation exposure since HSD11B1 expression is unchanged compared to the control group.

The data suggests that glucocorticoid level in MDS-5 Gy-MDS was substantially low at 2 days post-radiation, and it reflected in significantly lower HSD11B1 expression level which further support the protective effects of MDS on radiation-induced stress.

4.3.5 DNA Methyltransferase Family

It has been shown that radiation exposure could cause DNA hypomethylation. However, this effect is tissue and dose specific (78). In this study, adrenal tissue did not demonstrate any significant change in the expression levels of DNMT1,3A and 3B after 5Gy whole body radiation which could suggest a lower sensitivity of the adrenal tissue to radiation-induced changes in the expression levels of DNMTs (in the kidney tissue 5 Gy radiation caused significantly lower expression levels of DNMT3B).

Although 5Gy-MDS did not show any deviation from the expression pattern of the control group, MDS-5Gy-MDS demonstrated downregulation of DNMT3A by 120 days post-radiation. Previous studies provided positive effects of MDS on decreased baseline and

radiation-induced DNA damage shortly after radiation exposure (4 hour). However, not much data is available on MDS effects on DNMTs or DNA global methylation.

5.0 Conclusion and Future Directions

5.1 Overall Conclusion

Study of kidney tissue showed no radiation-induced change in RAS in the kidney tissue. On the other hand, data suggested presence of elevated levels of ROS considering the changes in expression levels of antioxidant enzymes such as GPX1, NOS3, and SOD2 in 5 Gy radiation group. MDS administration in combination with 5Gy radiation protects the kidney tissue from radiation-induced tissue damage by affecting two main targets: first, effectively reducing the activity of renin-angiotensin system (79) as it was predicted mainly by lower expression levels of angiotensinogen. MDS proved to be effective as prophylaxis (MDS-5Gy-MDS) or treatment (5Gy-MDS). Second, lowering the radiation-induced mitochondrial oxidative stress which was suggested by the study of NOS3, SOD1 and SOD2 expression patterns.

Radiation-induced physiological stress was manifested by elevated expression levels of PNMT, 2 days post-radiation in 5 Gy group. MDS was able to protect the mice from radiation-induced stress whether it was administered as prophylaxis or treatment. The activation of catecholamine biosynthesis response which was induced by radiation (2 days) was absent in animals treated by MDS. To provide more information on the diet's effects, systemic cortisol and epinephrine level could be studied. Looking at expression levels of genes in catecholamine biosynthesis pathway, 5 Gy-MDS group demonstrated a protection against radiation-induced physiological stress but in the long term showed no decrease in catecholamine biosynthesis pathway activity. On the other hand, considering the data suggesting lower expression levels of PNMT, DBH and TH in the MDS-5 Gy-MDS group in 30 and 120 days post-radiation if the lower catecholamine biosynthesis pathway activity

is in fact correlated with lower cortisone levels, it could provide patients with extra benefits (80).

5.2 Future Directions

To further investigate the data from current study and better interpret results from cellular mRNA levels I propose following experiments:

1- Study of protein levels and their active forms for the genes we already studied.

2- To address factors such as physiological stress closer time points (4-6 hours post-radiation) is suggested. In addition to mRNA levels, study of serum levels of cortisol and epinephrine by radioimmuneassay is also recommended.

3- To further investigate the DNA protection beside the proper time point (4 hours) tissue immunoassay is valuable to detect colonization of γH2AX to clarify and compare the DNA damage pattern in treatment groups.

4- Study ROS levels to provide further evidence for MDS protective effects against radiation-induced oxidative damage. Fluorogenic probes are used to study total ROS levels in an animal model cell lysate, serum or urine.

5- Study of organ specific RAS in tissues like brain and evaluation of MDS efficacy in protection against radiation-induced injury regarding local RAS function.

6- Study of proinflammatory and profibrotic cytokines at the said time points plus longer periods of time. Since inflammation is important in radiation-induced late effects study of these factors increases our knowledge on radioprotective effects of MDS.

5.3 Implication of current study in radiation field

Given the result from the current study and potential benefits of MDS in the protection of Tissue against cellular oxidative stress and physiological stress it is plausible that MDS could be utilized as a radiation protective agent in nuclear power workers or holders of any occupation with the possibility of incidental exposure to ionizing radiation like soldiers, pilots and astronauts. MDS has the potential to protect cellular function due to coverage of multiple targets in cells. Having that in mind MDS has a potential to be used in human diseases caused by oxidative stress like hypertension, diabetes, and cardiovascular diseases. It is also suggestible that MDS could be used to protect tissue from radiation injury as it happens due to cancer radiation therapy. However, a close study of MDS protective effects is needed to recognize whether MDS could selectively protect the normal tissue and not tumor cells.

6.0 References

1. Blakely EA. Biological Effects of Cosmic Radiation: Deterministic and Stochastic. Health Physics. 2000;79(5):495-506.
2. De Santis M DGE, Straface G, Cavaliere AF, Caruso A, Schiavon F, Berletti R, Clementi M. Ionizing radiations in pregnancy and teratogenesis. Reproductive Toxicology 2005;20(3):323-9.
3. Ziech D FR, Pappa A, Panayiotidis MI. Reactive Oxygen Species (ROS)—Induced genetic and epigenetic alterations in human carcinogenesis. Mutat Res. 2011;711(1-2):167-73.
4. Williams D. Radiation carcinogenesis: lessons from Chernobyl. Oncogene. 2009;27(Suppl 2):S9-S18.
5. Hall EJ, Giaccia, A.J. Linear Energy Transfer and Relative Biologic Effectiveness. In: Mitchell CW, editor. Radiobiology for the radiologists. 7 ed. USA: LIPPINCOTT WILLIAMS & WILKINS, a WOLTERS KLUWER; 2012.
6. Hall EJ, Giaccia, A.J. Doses and Risks in Diagnostic Radiology, Interventional Radiology and Cardiology, and Nuclear Medicine. In: Mitchell CW, editor. Radiobiology for the radiologists. USA: LIPPINCOTT WILLIAMS & WILKINS, a WOLTERS KLUWER; 2012.
7. Hall EJ, Giaccia, A.J. Physics and Chemistry of Radiation Absorption. In: Mitchell CW, editor. Radiobiology for the radiologists 7ed. USA: wolters kluwer-lippincott williams & wilkins; 2012. p. 3-11.
8. Rubin P JC, Williams JP, McDonald S, Finkelstein JN. Perpetual cascade of cytokines post-irradiation leads to pulmonary fibrosis. Int J Radiat Oncol Biol Phys. 1995;33(1):99-109.
9. Chen Y WJ, Ding I, Hernady E, Liu W, Smudzin T, Finkelstein JN, Rubin P, Okunieff P. Radiation pneumonitis and early circulatory cytokine markers. Semin Radiat Oncol. 2002;12(1):26-33.
10. Stone HB CC, Anscher MS, McBride WH. Effects of radiation on normal tissue: consequences and mechanisms. The Lancet. 2003;4(9):529-36.
11. Brush J LS, Phillips T, Sitko J, McDonald JT, McBride WH. Molecular Mechanisms of Late Normal Tissue Injury. Radiation oncology. 2007;17(2).
12. Dörr W HJ. Consequential late effects in normal tissues. Radiother Oncol. 2001;61(3):223-31.
13. Denham JW H-JM. The radiotherapeutic injury – a complex 'wound'. Radiother Oncol. 2002;63(2):129-45.
14. PA R. Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol. 1994;65(1):27-33.
15. Mikkelsen RB WP. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003;22(37):5734-54.
16. Azzam EI J-GJ, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Letters. 2012;327(1-2).
17. Somosy Z. Radiation response of cell organelles. Micron. 2000;31(2):165-81.
18. R LE. Angiotensin and aldosterone. Regulatory Peptides 1999;80(3):91-100.
19. Brewster UC PM. The Renin-Angiotensin-Aldosterone System and the Kidney: Effects on Kidney Disease. The American Journal of Medicine. 2004;116(4):263-72.

20. Mitchell KD BB, Navar LG. Hypertensinogenic Mechanisms Mediated by Renal Actions of Renin-Angiotensin System. Hypertension 1992;19(1 Suppl).
21. Atlas SA. The Renin-Angiotensin Aldosterone System: Pathophysiological Role and Pharmacologic Inhibition. J Manag Care Pharm. 2007;13(8 Suppl B).
22. Molteni A MJ, Cohen EF, Ward WF, Fish BL, Taylor JM, Wolfe LF, Brizio-Molteni L, Veno P. Control of radiation-induced pneumopathy and lung fibrosis by angiotensin-converting enzyme inhibitors and an angiotensin II type 1 receptor blocker. Int J Radiat Biol. 2000;76(4):523-33.
23. Zhao W1 DD, Robbins ME. Oxidative damage pathways in relation to normal tissue injury. Br J Radiol. 2007;80(1).
24. Lee WH WJ, Sonntag WE, Lee YW. Irradiation alters MMP-2/TIMP-2 system and collagen type iv degradation in brain. Int J Radiat Oncol Biol Phys. 2012;82(5).
25. Moulder JE FB, Cohen EP. ACE inhibitors and AII receptor antagonists in the treatment and prevention of bone marrow transplant nephropathy. Curr Pharm Des. 2003;9(9):737-49.
26. Robbins ME DD. Pathogenic role of the renin-angiotensin system in modulating radiation-induced late effects. Int J Radiat Oncol Biol Phys. 2006;64(1).
27. Hall EJ, Giaccia, A.J. Clinical Response of Normal Tissues. In: Mitchell CW, editor. Radiobiology for the radiologists USA: Lippincott Williams & Wilkins, a Wolters Klumer; 2012.
28. Cohen EP RM. radiation nephropathy. Semin Nephrol. 2003;23(5):486-99.
29. Cohen EP FB, Moulder JE. The renin-angiotensin system in experimental radiation nephropathy. J Lab Clin Med. 2002;139(4):251-7.
30. Brach MA HR, Sherman ML, Gunji H, Weichselbaum R, Kufe D. Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. J Clin Invest. 1991;88(2):691-5.
31. FC L. Proinflammatory effects of angiotensin II and endothelin: targets for progression of cardiovascular and renal diseases. Curr Opin Nephrol Hypertens. 2002;11(1):59-66.
32. Smith SM VW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues Clin Neurosci. 2006;8(4):383-95.
33. Ullian ME. The role of corticosteroids in the regulation of vascular tone. Cardiovascular research. 1999;41:55-64.
34. Wong DL TT, Wong-Faull DC, Claycomb R, Kvetnansky R., information A. Genetic Mechanisms for Adrenergic Control during Stress. Ann NY Acad Sci. 2004;1018:387-97.
35. Kakiki M MK, Nomura M, Omura T, Horie T. Expression of aldosterone synthase cytochrome P450 (P450aldo) mRNA in rat adrenal glomerulosa cells by angiotensin II type 1 receptor. Endocr Res. 1997;23(4):227-95.
36. Siegel GJ. Biosynthesis of Catecholamines. In: Siegel GJ AB, Albers RW, et al editor. Basic Neurochemistry: Molecular, Cellular and Medical Aspects: Lippincott-Raven; 1999.
37. Donal L Wong TCT, David C Wong-Faull, Rbert Claycomb, Richard Kvetnansk. Genetic Mechanisms for Adrenergic Control during Stress. Annals of the New York Academy of Sciences. 2004;1018:387-97.
38. Deborah Citrin. Ana P. Cotrim FH, Bruce J. Baum, Muralic C. Krishna, James B. Mitchell. Radioprotectors and Mitigators of Radiation-Induced Normal Tissue Injury. The Oncologist 2010;15.

39. Paul Okunieff SS, Peter Keng, Weimin Sun, Wei Wang, Jung Kim, Shanmin Yang, Hengshan Zhang, Chaomei Liu, Jacqueline P. Williams, Amy K. Huser, and Lurong Zhang. Antoxidants reduce consequences of radiation exposure. Adv Exp Med Biol. 2008;614.
40. Lemon JA BD, Rollo CD. A dietary supplement abolishes age-related cognitive decline in transgenic mice expressing elevated free radical processes. Exp Biol Med (Maywood). 2003;228(7):800-10.
41. Lemon JA BD, Rollo CD. A complex dietary supplement extends longevity of mice. J Gerontol A Biol Sci Med Sci. 2005;60(3).
42. Lemon JA RC, Boreham DR. Elevated DNA damage in a mouse model of oxidative stress: impacts of ionizing radiation and a protective dietary supplement. Mutagenesis. 2008;23(6).
43. Aksenov V LJ, Lokuge S, Foster JA, Liu J, Rollo CD. Dietary amelioration of locomotor, neurotransmitter and mitochondrial aging. Exp Biol Med (Maywood). 2010;235(1).
44. J.A.Lemon CDR, D.R.Boreham. Elevated DNA damage in a mouse model of oxidative stress: impacts of ionizing radiation and a protective dietary supplement. Mutagenesis. 2008;23(6).
45. Fraga D, Meulia,T.,Fenster, S. Real-Time PCR. Current Protocols Essential Laboratory Techniques. 2008:10.3.1-.3.33.
46. Moulder JE CE. Future strategies for mitigation and treatment of chronic radiation-induced normal tissue injury. Semin Radiat Oncol. 2007;17:141-8.
47. AB R. Angiotensin II antagonists-therapeutic benefits spanning the cardiovascular disease cintinuum from hypertension to heart failure and diabetic nephropathy. Curr Med Res Opin. 2006;22(1):1.
48. Robbins ME DD. Pathological role of the renin-angiotensin system in modulating radiation-induced late effects Int J Radiat Oncol Biol Phys. 2006;64(1).
49. J. Racquel Collins-Underwood WZ, Jessica G. Sharpe, and Mike E Robbins. NADPH oxidase mediates radiation-induced oxidative stress in rat
brain microvascular endothelial cells. Free Radic Biol Med 2008 September 15; 45(6): 929–938. 2008;45(6).
50. Kohara K BK, Ferrario CM, Milsted A. Peripheral and central angiotensin II regulates expression of genes of the renin-angiotensin system Am J Physiol. 1992; 262 (5 Pt 1):E651-E7.
51. Snyder R TT. Interplay between EGR1 and SP1 is critical for 13-cis retinoic acid-mediated transcriptional repression of angiotensin type 1A receptor. Research. 2013;50(3):361-74.
52. Nguyen T NP, Pickett CB. The Nrf2-Antioxidant Response Element Signaling Pathway and Its Activation by Oxidative Stress. J Biol Chem. 2009;284(20):13291-5.
53. Kwak MK IK, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the Nrf2 promoter. Mol Cell Biol. 2002;22(9):2883-92.
54. Baud O GA, Li J, Wang H, Volpe JJ, Rosenberg PA. Glutathione Peroxidase–Catalase Cooperativity Is Required for Resistance to Hydrogen Peroxide by Mature Rat Oligodendrocytes. J Neurosci. 2004;24(7):1531-40.
55. Rabender CS AA, Mikkelsen RB. Ionizing Radiation Induced Nitric Oxide Signaling. Austin J Nucl Med Radiother. 2014;1(1):1-5.

56. Pacher P BJ, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87(1):315-424.
57. Long J AV, Rollo CD, Liu J. A complex dietary supplement modulates nitrative stress in normal mice and in a new mouse model of nitrative stress and cognitive aging. Mech Ageing Dev. 2012;133(8).
58. Mikkelsen RB WP. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003 22(37):5734-54.
59. Dikalov SI NR. Angiotensin II-Induced Production of Mitochondrial Reactive Oxygen Species: Potential Mechanisms and Relevance for Cardiovascular Disease. Antioxid Redox Signal. 2013;19(10).
60. Barjaktarovic Z SD, Shyla A, Azimzadeh O, Schulz S, Haagen J, Dörr W, Sarioglu H, Schäfer A, Atkinson MJ, Zischka H, Tapio S. Radiation–Induced Signaling Results in Mitochondrial Impairment in Mouse Heart at 4 Weeks after Exposure to X-Rays. Plos One. 2011;6(12):1-15.
61. Aksenov V LJ, Liu J, Szechtman H, Khanna P, Matravadia S, Rollo CD. A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. Age (Dordr). 2013;35(1).
62. Schraufstatter IU HP, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG. Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. Proc Natl Acad Sci U S A. 1986;83(13):4908-12.
63. Dizdaroglu M, Nackerdien,Z., Chao,B.C., Gajewski,E. and Rao,G. . Chemical nature of in vivo DNA base damage in hydrogen peroxide-treated mammalian cells. Arch Biochem Biophys. 1991;285(2):388-90.
64. Aziz Sancar LAL-B, Tae-Hong Kang, Joyce T. Reardon, Jin Hyup Lee, Nuri Ozturk. Circadian clock control of the cellular response to DNA damage. FEBS Letters 2010;584(12).
65. David Miguel SP, Andrew Flaus. Genome Stability and Human Diseases. Structure and Function of Histone H2AX. 50: springer science+business media; 2009. p. 55-78.
66. J E Kalinyak CAG, R W Hamilton, J G Bradshaw, A J Perlman, and A R Hoffman. Developmental and hormonal regulation of glucocorticoid receptor messenger RNA in the rat. J Clin Invest. 1989;84(6):1843–8.
67. Tawa R KY, Komura J, Miyamura Y, Kurishita A, Sasaki MS, Sakurai H, Ono T. Effects of X-ray irradiation on genomic DNA methylation levels in mouse tissues. J Radiat Res. 1998;39(4):271-8.
68. Pogribny I RJ, Slovack M, Kovalchuk O. Dose-dependence, sex- and tissue-specificity, and persistence of radiation-induced genomic DNA methylation changes. Biochem Biophys Res Commun. 2004;320(4):1253-61.
69. Szyf M. DNA Methylation and Cancer Therapy.
70. Azhar S CL, Reaven E. Alteration of the Adrenal Antioxidant Defense System During Aging in Rats. J Clin Invest. 1995;96(3):1414-24.
71. Sabban EL KR. Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. Trends Neurosci. 2001;24(2):91-8.
72. Viskupic E KR, Sabban EL, Fukuhara K, Weise VK, Kopin IJ, Schwartz JP. Increase in rat adrenal phenylethanolamine N-methyltransferase mRNA level caused by immobilization stress depends on intact pituitary-adrenocortical axis. J Neurochem. 1994;63(3):808-14.

73. Shih J. Stress-Induced Expression of Phenylethanolamine N-Methyltransferase: Normal and Knock out Animals. In: McCarty RA, G.,Sabban, E.L., Kvetnansky, R., editor. Stress: neural, endocrine, and molecular studies2002. p. 129-35.
74. Naoi Mea. Stress induced changes in TH and other catecholamine synthesizing enzyme. In: Kvetnv anský RaS, E. L., editor. Tyrosine Hydroxylase: VSP Press, International Science Press; 1993. p. 253-81.
75. Sabban EL HB, Nankova B, Kvetnanský R. Molecular biology of stress-elicited induction of catecholamine biosynthetic enzymes. Ann N Y Acad Sci. 1995;771:327-38.
76. W. Feldberg GPL. The action of peptides on the adrenal medulla. Release of adrenaline by bradykinin and angiotensin. J Physiol. 1964;171(1): 98–108.
77. Boadle-Biber MC HJ, Roth RH. Acceleration of catecholamine biosynthesis in sympathetically innervated tissues by angiotensin-II-amide. Br J Pharmacol 1972;46(2):289-99.
78. , Karen Chapman MH, Jonathan Seckl. 11β-Hydroxysteroid Dehydrogenases: Intracellular Gate-Keepers of Tissue Glucocorticoid Action. Physiol Rev. 2013;93(3).
79. Lee Y KY, Choi YJ, Lee JW, Lee S, Cho YH, Chung HW. Radiation-induced changes in DNA methylation and their relationship to chromosome aberrations in nuclear power plant workers. Int J Radiat Biol. 2015;91(2).
80. Kitiyakara C CT, Chen Y, Blau J, Karber A, Aslam S, Welch WJ, Wilcox CS. Salt Intake, Oxidative Stress, and Renal Expression of
NADPH Oxidase and Superoxide Dismutase. J Am Soc Nephrol 2003;14(11):2775-82.
81. Myrthala Moreno-Smith SKL, Anil K Sood. Impact of stress on cancer metastasis