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Does Spaceflight Increase the Chance of Female Astronauts Developing Uterine Cancer?

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Does Spaceflight Increase the Chance of Female Astronauts Developing Uterine Cancer?

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ABSTRACT

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One of the main questions put forth by NASA and the European Space Agency (ESA) is whether or not an organism, especially mankind, can complete an entire life cycle in space. With this in mind, it is essential to study the effect of spaceflight on reproductive tissues. Using simulated microgravity techniques and whole-body radiation we sought to determine if females subjected to a simulated spaceflight environment have increased incidences of uterine cancer. Uterine tissue from mice subjected to simulated spaceflight was analyzed using immunohistochemical staining and western blot analysis. Two pathways commonly activated in cancer were investigated. Additionally, the uterine tissue was evaluated for gross morphological changes using standard histological staining. The findings of this study indicate that none of the treatment parameters used to simulate the spaceflight environment were found to induce uterine cancer.

DEDICATION

I dedicate this work to my family for the love, endless support, and encouragement which helped me in completion of this thesis. My beloved and supportive husband, Issa, who is always by my side when times I needed him most and my lovable baby, Karam, who served as my inspiration to continue this undertaking. I thank them both for their continued unconditional support and sacrifices.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

One of the main questions put forth by NASA and the European Space Agency (ESA) is whether or not an organism, especially mankind, can complete an entire life cycle in space. With this in mind, it is essential to study the effect of spaceflight on reproductive tissues as one of the significant human organs. There are two factors involved with spaceflight that are dramatically different from conditions on Earth. First, the spaceflight environment is one of very low gravity (microgravity). Second, the spaceflight environment is one of relatively high levels of radiation (galactic cosmic radiation) compared to that on Earth. Spaceflight has been shown to have effects on many different tissues of the body (Williams et al. 2009). It is unknown whether those effects are due to the microgravity of spaceflight, the elevated levels of radiation, or a combination of the two.

The primary physiological responses of vertebrates to spaceflight microgravity include fluid shifts, cardiovascular problems, musculoskeletal unloading, disturbance of senses, and immunity dysregulation (Pace 1977; Vernikos 1996).

Effect of Microgravity on the Cardiovascular System

Compromised cardiovascular functions have been reported in astronauts during spaceflight. Orthostatic intolerance and reduced aerobic capacity seemed to be the most pressing of cardiovascular issues faced by astronauts (Convertino 2009). Orthostatic intolerance is a serious health problem that faces astronauts during re-entry into earth's atmosphere and immediately after landing. Orthostatic intolerance is characterized by inability to remain in upright posture while maintaining adequate blood supply to the brain. One hypothesis that can describe these changes is cephalad fluid shift (Charles and Lathers 1991). Normally on earth and under the effect of 1g gravity, the hydrostatic pressure is higher in the lower body compartment

than the upper body. This is because the fluid in the upper part of the body exerts a pressure on the fluid in the lower part of the body. Venous return is the volume of blood in systemic veins that flow back to the heart. In a standing position, the return of venous blood to the upper part of the body is challenged. This is because the blood flow has to overcome the effect of gravity. The skeletal muscle pump helps force the blood toward heart. The peripheral veins in the legs have one-way valves that do not allow backflow of blood away from the heart. These valves counteract the gravitational pull and prevent backflow of blood when standing. Upon entering the space environment, the skeletal muscle pump is still pushing the blood in a cephalad direction, but there is no resistance to this blood flow because there is no gravitational force pulling the blood back down. This fluid shift toward the head (cephalad) is called cephalad fluid shift (Drake et al. 2015).

Microgravity initiates a fluid shift, due to absence of hydrostatic pressure gradients, with blood and other fluids moving from lower limbs toward head and thorax. Facial swelling is a common change in astronauts due to high blood supply in head region. During spaceflight, the volume in lower limbs is reduced by 10% compared with preflight (Leach et al. 1991). Earth based models which are used to study these changes showed that cephalad fluid shifts distend baroreceptors of the central vasculature. This triggers the suppression of the renin-angiotensin-aldosterone system (RAAS). Suppression of the RAAS leads to increased renal excretion of salt and water, and a net reduction in plasma volume (Vernikos 1995). Changes in the vestibular system, especially in processing inputs from otolith receptors, is another hypothesis to describe orthostatic intolerance. Altered gravity produces significant changes in the vestibular system and this leads to changes in sympathetic nervous system activity including blood pressure regulation (Yates and Kerman 1998). Further investigation is required to explain the relation between vestibular changes and orthostatic intolerance.

Musculoskeletal Changes in Microgravity

The effect of actual and simulated microgravity on the musculoskeletal system is well documented. Absence of gravitational loading during spaceflight leads to muscle atrophy and bone demineralization. Weightlessness was shown to lead to changes in muscle structure and function. These changes appear mainly as a decline in muscle mass, volume, and strength (di Prampero and Narici 2003). Muscle atrophy mainly occurs due to imbalance in protein synthesis and degradation. In general, there is an increase in protein degradation and a decrease in protein synthesis, and loss of neuromuscular control. These causes are attributed to loss of gravitational loading. The most affected muscles are postural muscles, such as those found in the calf and thigh, that maintain our bodies in an upright position in a gravitational environment (Clément 2011).

Bone demineralization is a well-known health consequence of long duration spaceflight which was recognized even long before the first manned space mission. The loss of bone mass during spaceflight could eventually weaken the bone and increase the risk of fracture and so presents problems to astronauts upon return to a normal gravity environment. Studies of astronauts who spent months on space station Mir and Skylab, and also ground based models simulating microgravity, all showed that astronauts could lose 1-2% of bone mass. This loss occurred predominantly in load bearing areas of legs and lumbar spine (Vico et al. 2000; Lang et al. 2004; LeBlanc et al. 2007). Data from space flight studies showed increased urinary and fecal excretion of calcium and increased markers of bone resorption. Calcium concentration is controlled mainly by parathyroid hormone (PTH) and calcitonin. PTH is responsible for bone resorption and thus activated when the blood calcium concentration is low. Normally, serum calcium and PTH are negatively correlated: when the level of calcium in the blood is low, the concentration of PTH will be high in response. Reduced levels of parathyroid hormone and

vitamin D in astronauts are expected physiologic responses to increased blood calcium resulting from microgravity-induced bone loss (Smith and Heer 2002). One possible explanation for this bone loss is the reduced gene expression of systemic hormones and growth factors (such as collagen Iα1, alkaline phosphatase, and osteocalcin) that are considered important for osteoblast differentiation (Carmeliet et al. 1998). Other studies showed a several fold increase in genes involved in osteoclast activity and maturation (Tamma et al. 2009). Impaired osteoblast function and increased osteoclast activity could explain osteoporosis like symptoms associated with space flight.

The Effect of Microgravity on Immune System

There is strong evidence associated with impaired immune system functions and space flight. Immune dysregulation in astronauts was first observed in the 1960s and 1970s as increased incidences of bacterial and viral infections were reported during or immediately after space flight. Fifteen of 29 Apollo crew members developed bacterial or viral infections during their missions or within the first week after their return (Hawkins and Zieglschmid 1975). Much is known about the immune health of astronauts from post flight testing of crew members, but little data are available about immunity during space flight. More in-flight investigation using human subjects is needed. These alterations of immune function could be attributed to high levels of physiological and physical stress during and immediately after space flight. Factors like radiation, microgravity, and altered sleep cycle could all affect the astronaut's immune system. The few in flight studies that have been conducted have shown reactivation and increased frequencies of Epstein bar virus, varicella zoster virus and cytomegalovirus (Mehta et al. 2000, 2003; Pierson et al. 2005). Reduced cell mediated immunity is also reported in in-flight studies (Gmünder et al. 1994). Post flight studies have revealed depression of T cell function during long duration flight, altered cytokine production, reduced function of natural killer cells and

granulocytes, and latent viral reactivation (Crucian et al. 2009). Altered cytokine production and potential shifts to a T-helper 2 (Th2) pattern lead to immune imbalance and adverse health conditions. For example, depressed immune response can result in increased incidence of infection, but heightened immunity may lead to allergies and autoimmunity. Microgravity could be associated with some impairments of immunity. In flight and ground-based models have shown that weightlessness impairs signal pathways essential for early T cell activation (Boonyaratanakornkit et al. 2005). Potential negative health consequences of prolonged immune dysregulation include hypersensitivity, autoimmunity, infectious diseases and even malignancies. So, it is important to determine the clinical risk of impaired immunity before initiating long missions to space.

Genitourinary Problems During Spaceflight

The genitourinary (GU) system is not uncommonly affected during space flight. Almost 50 astronauts out of 508 crew members between 1981 and 1998 reported GU symptoms during space flights (Jones et al. 2005). GU symptoms can be categorized as: upper tract, lower tract and reproductive health. GU issues of upper tract during space flight are calculi and related obstruction. Data collected during short term space flight, long duration Mir missions and immediately after space flight have revealed increased urinary calcium and a decrease in both urinary volume and urinary pH resulting in a great risk of kidney stone formation (Whitson et al. 1999). As mentioned earlier, bone loss associated with space microgravity lead to increased excretion of calcium by the kidneys and increased the urinary supersaturation of stone forming salts. Several cases of urinary tract infection and retention have also been reported during space flight. Multiple causative factors could be attributed to urinary tract infection. Stresses, dehydration, hygiene, and delayed access to voiding could play a role (Whitson et al. 1999).

Effective countermeasures should be taken since these issues don't affect astronaut's health only, but also affect the success of missions.

With increased durations of space flight missions, an understanding of sex/ gender effects on reproductive health is needed. Major factors in the space environment that could affect reproductive health are: radiation, microgravity, and stresses. A 120-day Russian bed rest study found reduce numbers of active spermatozoa collected after 50-60 and 100 days of the study, and also an increase in the percentage of morphologically altered spermatozoa (Nichiporuk et al. 1998). One ground-based study of male rats exposed to long duration simulated microgravity by hindlimb unloading (HU) for 6 weeks reported a significant decline in testicular weights and spermatogenesis, no spermatogenic cells were present and epididymides were devoid of mature sperm (Tash et al. 2002). Another HU study documented adverse effects on the tissue that comprise the walls of the seminiferous tubules and the blood-testis barrier, with these changes noticeable after only 7 days of HU (Forsman 2012).

With increased involvement of female astronauts in the space program including long duration space missions, more investigation is required to address the effects of the spaceflight environment on the female reproductive system. Because female astronauts use hormone supplements to suppress their menstrual cycles during spaceflight, reproductive changes in female astronauts have not been studied during or after space flight. The effects of spaceflight microgravity on menstrual functioning and microgravity induced retrograde menstruation need to be studied.

Animal studies have provided data on some of the female reproductive changes associated with exposure to microgravity. Female rats exposed to simulated microgravity using the HU model have shown lengthening of diestrus phase of the reproductive cycle and therefore

lengthening of the estrous cycle (Tou et al. 2004). A prolonged diestrus cycle could result in reduction in the cumulative number of cycles and thus compromise fertility. Another study used 2g centrifugation to study the effects of hypergravity on the female reproductive system of rats. This study found delayed conception in centrifuged rats compared to non-centrifuged rats. This study also showed that both centrifuged and non-centrifuged rats conceived, but centrifuged females took 3 times longer than non-centrifuged females to achieve conception (Ronca et al. 2005).

A study by Kojima et al. (2000) looked for the effect of simulated microgravity on mammalian fertilization and embryonic development in vitro using clinorotation. In vitro fertilization of 80 oocytes and 1x 10^6 sperm/ml were cultured in a microtube under clinostat rotation and stationary control conditions. Their results indicated that fertilization can occur in a simulated microgravity condition. But there was a significant decrease in the number of embryos reaching the morula and blastula stages after 96 hours of embryos culture under clinostat rotation. Another study investigated the effect of spaceflight on the event of parturition in female rats. These female rats were in mid pregnancy when flown on either 9 or 11 day long spaceflights. Each shuttle landed just 2-3 days before parturition. They found that flight female rats had uncomplicated successful vaginal delivery and delivery occurred at the appropriate gestational time, however the flight rats had twice the number of lordosis contractions compared with control group (Ronca and Alberts 2000).

More recent studies have addressed the effect of microgravity on the mammalian female reproductive system. Mice that were flown for 13 days on the space shuttle Endeavor had a trend toward thinning of the mucin layer in the uterine tube compared with control group (Svalina and Forsman 2013). Uterine tissues of female mice flown on the NASA shuttle mission STS-118 had significantly thicker apical mucin layer than control groups (Forsman and Near 2013). The

thicker mucin layer in flight animals could be attributed to the effect of spaceflight on the pituitary- gonadal axis which could interfere with LH and FSH production. However, Smith and Forsman (2012) conducted a study to evaluate the morphological features of the ovaries of these same animals and found no gross morphological differences in follicle counts and number of corpora lutea between flight and control animals. Another study found that female mice who were exposed to 13 days of spaceflight had a thicker layer of mucin lining the vaginal canal than control mice (Romer and Forsman 2015). According to this study, spaceflight could have negative effects on sperm survival and may compromise the animal's ability to reproduce. Although changes in mucin thickness were documented in these studies, it was unclear whether or not these changes were due to the microgravity of spaceflight, the radiation of spaceflight, or a combination of the two.

Ground Based Models to Simulate Microgravity

There are different earth-based techniques used to simulate microgravity and predict some effects of spaceflight on different body organs and systems. One example of these techniques is clinorotation via a device known as a clinostat. This device uses rotation to constantly change the gravitation vector so that the vectors cancel each other rendering no gravitational vector (Klaus 2001). Other examples are head-down bed rest and water immersion of humans. The last two techniques mimic the microgravity component of spaceflight because they induce cephalad fluid shifts and loss of loading on the musculoskeletal system (Epstein 2011; Fortney et al. 2011). The HU model of rodents is the newest technique to simulate microgravity and weightlessness and it's the technique used in our experiment so I will explain this technique in the following paragraph. These earth-based models are very beneficial in space exploration for several reasons: they are cost effective techniques, provide critical data without consuming valuable crew time, manipulations can be made with little to no cost increase, and

allow for variable experimental duration with multiple time points being measured throughout the experiment. Moreover, unlike spaceflight studies, the experiment can be repeated or extended as needed and tissues taken from the animal at any time during the experiment.

The HU model has been accepted by the scientific community as the rodent model of choice to study the adverse effects of spaceflight microgravity and weightlessness on various body systems, especially the musculoskeletal system. The standard operating procedure for this technique was updated and approved by the National Aeronautics and Space Administration (NASA), Ames Research Center (ARC), and Flight Institutional Animal Care and Use Committee (Flight IACUC) on August 8, 2001. The development of HU model began in 1975 with the results from un-manned spaceflight experiments compared with a HU model. COSMOS 2044, was the first international spaceflight mission that include the HU model in the experiment as control group (Grindeland et al. 1992; Booth and Grindel 1994). Both spaceflight and the HU model showed similar but not identical physiological responses in most of the body organs (Vico et al. 1991; Chapes et al. 1993; Tischler et al. 1993). The physiological responses that the HU model has to mimic include 1) differential muscle atrophy, 2) cephalad fluid shift, 3) freedom to move and eat using the forelimbs, and 4) unloading of the hindlimbs without paralysis so that animals can recover from unloading (Morey-Holton and Globus 2002). There are many differences between HU simulated microgravity and actual spaceflight microgravity, therefore care should be taken when comparing results between spaceflight and HU experiments. The entire body is unloaded during spaceflight, while the forelimbs are weight bearing in the HU model. Therefore, responses in the HU model reflect partial effects of unloading of the musculoskeletal system on mammalian fertilization and embryonic development. Finally, the HU group does not experience launch, landing, or transportation to and from the launch and landing pad.

The HU technique as provided by NASA ARC is as follows. HU depends on using tail traction, surgical pins, or a back harness to unload the hindquarters of rats or mice. This causes cephalad fluid shifts and removal of gravity forces from hindlimbs, similar to what is seen in astronauts. Tail traction is used more in the HU model since it is associated with less stress and there is no need for anesthesia. Handling of the animals begins 1 week before the procedure. The animals are acclimated to the cages at least 2 days before unloading. It is recommended to avoid anesthesia, since recovery from anesthesia may affect the animal's ability to adapt to HU. If anesthesia is used, then a 24-hour recovery period is needed before the procedure. The procedure begins with using alcohol to clean the tail and remove dirty or dead skin. The tail is allowed to dry, then sprayed with strong adhesive and allowed to dry for 1-5 minutes. When the spray is tacky, a piece of traction tape that was attached to the plastic tab, is attached to the tail, just above the hair line. The traction tape is then gently pressed to stick to the surface of the tail. Two strips of filament tape are used to secure the traction tape. One of the filament tape strips is secured around the edge of the traction tape at base of the tail, the second strip is placed about half-way up the traction tape. The filament tape should be loose enough to allow for adequate blood circulation, while still preventing the traction tape from peeling away from the tail. Gauze bandage can be wrapped around the base of the tail to protect the traction tape and to prevent the animal from peeling off the tape. A paper clip is then used to attach the animal to a fish-line swivel that hangs from the unloading device on top of the HU cage. The swivel is designed to prevent only the back feet from touching the cage's floor while allowing the animal to move freely using the forelimbs. The animal's body makes about a 30° angle with the floor of the cage. A 30° angle of unloading between the cage floor and the torso of the animal is the optimum degree to simulate spaceflight microgravity as it provides normal weight bearing on the forelimbs and induces cephalad fluid shifts. As the angle increases, mechanical loading of the

forelimbs declines and traction on the tail increases. If the angle is too steep, the animals appear stressed. The cage sides can be adjusted to control the height of the unloading device and allow the animal to reach food and water. The animal's overall appearance, activity, and the tail should be inspected daily to assure the health of the animals

The Nature of Space Radiation

Space radiation consists mainly of three types of radiation: particles trapped in Earth's magnetic field, energy from solar particle events, and galactic cosmic rays (GCRs) (NASA Facts. National Aeronautics and Space Administration, 2002). GCRs are the primary type of space radiation that astronauts are exposed to on the international space station (ISS), and also on future space missions. GCRs are considered ionizing radiation. Opposite to non-ionizing radiation, ionizing radiation has enough energy to knock the electrons out of any atoms it strikes causing biological changes of exposed cells and tissues. Astronauts are exposed to approximately 50-2000 milliseivert (msv) in 6 months depending if they are on the ISS or the surface of moon or Mars (Barcellos-Hoff et al. 2015). Since radiation effect studies are difficult to carry out on human cells and tissues, the risks associated with radiation exposure should be obtained from ground-based studies using animal models. Ground based studies of radiobiological effects are largely limited to studies involving survivors of nuclear bombing events in Japan during world war II, cancer survivors who were exposed to radiation as part of their cancer treatment, and most recently people exposed to nuclear radiation resulting from nuclear disasters like ones in the Chernobyl nuclear power plant in Ukraine and the Fukoshima nuclear disaster in Japan. (Thompson et al. 1994; Cardis et al. 2006; Dörr and Meineke 2011).

Sources and Effects of Radiation Exposure

Radiation is all around us. The sources for ionizing radiation could be natural or man-made. Natural sources of radiation include: cosmic radiation and terrestrial radiation. Terrestrial

radiation is present in Earth's crust in natural deposits of uranium and potassium. Natural sources could also be through inhalation of radioactive gases, such as radon, from the earth soil and minerals or through ingestion of trace amounts of radiation from some food contents or ground water (Washington State Department of Health 2002). Some man-made sources of radiation are used for medical purposes. The best-known application of radiation in the medical field are X-ray machines for medical imaging and diagnosis and nuclear medicine which uses radioactive isotopes to treat cancer (Canadian Nuclear Safety Commission 2012).

Radiation has been shown to have harmful effects on human health. The effect of ionizing radiation on human health depends on the total dose, exposure rate, and type of tissues exposed to radiation. The highest effect will be when exposure occurs in large doses over a short period of time. Some cells are more radiosensitive than others. Cells that are immature, undifferentiated, and have higher mitotic activity are more radiosensitive than mature, non-dividing cells (Goodman 2010). Examples of radiosensitive tissues are blood cells, gonads, lens of the eye, and skin.

The effect of ionizing radiation on the cellular level can be divided into direct and indirect damage. DNA is the major target in radiation damage. Ionizing radiation produces a wide range of DNA lesions, such as base damage, SSBs, double strand breaks (DSBs), and DNA-DNA and DNA-protein crosslinks (Prise et al. 2005). Base damage and SSBs' repair occur via base excision repair and DNA ligation respectively. DSBs have been thought to be the most relevant lesion for cell killing. Homologous recombination (HR) and non-homologous end joining (NHEJ) are common repair pathways for DSBs (Jackson 2002). NHEJ is a dominant pathway in mammalian cells and it is more error prone than HR. Incorrectly repaired or unrepaired lesions can lead to DNA mutations or cell death. Cell death can occur through activation of programmed cell death pathways (such as apoptosis and autotrophy) or necrosis

(Brown and Rzucidlo 2011). Radiation induced apoptosis occurs through intrinsic pathways which involve changes in mitochondria or through activation of caspase enzymes that specifically cleave proteins (Prise et al. 2005).

Absorption of ionizing radiation by cells can generate reactive chemical species (reactive oxygen species, reactive nitrogen species) that may cause damage to DNA, proteins, and lipids. This effect of radiation is called indirect effect of radiation and it contributes to more damage than the direct effect. Because 80% of cellular content is water, ionizing radiation can cause hydrolysis of water to produce hydrogen molecules and hydroxyl free radical (.OH). Hydroxyl free radical has an unbound electron and therefore higher affinity to react with other molecules. When two hydroxyl molecules bind to each other, they form hydrogen peroxide which is highly toxic (Brown and Rzucidlo 2011).

There are two types of radiation induced injury, stochastic and deterministic (Brown and Rzucidlo 2011). Deterministic injury occurs once the threshold of radiation exposure has been exceeded. The severity of deterministic injury increases as the dose increases. Examples of deterministic effects include skin erythema, cataracts, epilation, and sterility. Acute radiation syndrome is a form of deterministic injury that occurs when exposed to large doses of radiation in a short period of time. Signs and symptoms of acute radiation syndrome begin with gastrointestinal symptoms, nausea, vomiting, diarrhea, and fluid loss. Neurological damage may occur in larger doses (Dörr and Meineke 2011).

Stochastic effects or long-term effects of radiation are those effects that could appear years after radiation exposure. These late effects of radiation may result from previous high dose exposure or from chronic low dose exposure over years. Long term effects could appear as increased incidence of cancer or hereditary defects. The first reported increase in cancer risk

associated with radiation exposure was a report in 1944 of a 10-fold increase in leukemia among radiologists (March 1994). Most of the data about radiation induced cancer comes from epidemiological studies in human populations receiving radiation exposure from occupational, medical, and accidental sources. An epidemiological study done 20 years after the Chernobyl accident showed a dramatic increase in incidence of thyroid cancer (Cardis et al. 2006). Another epidemiological study on Hiroshima and Nagasaki atomic bomb survivors showed significant increases in solid tumors in most tissues (Thompson et al. 1994).

RIBE stands for radiation induced bi-standard effect, and is another effect of radiation. RIBE challenged the conventional dogma that radiation affects only the exposed cells and does not have any effects on non-exposed cells. RIBE can be defined as the phenomenon in which irradiated cells can release signaling molecules, which transfer via gap junctions to non-irradiated cells and cause similar toxicity or genetic damage (Han and Yu 2010). By looking at the radiation effects from epidemiological studies and cancer survivors, we can predict similar or more sever effects from galactic cosmic radiation on various body organs depending on the duration the astronauts spend on the international space station, or the surface of the moon or Mars.

Effect of Radiation on Reproductive Health

Ionizing radiation has effects on gonads. The degree of damage depends on many factors such as the dose, duration and age of exposure (Goodman 2010). The testis is one of the most radiosensitive tissues. Radiation exposure to testis occurs through therapeutic, diagnostic, and occupational exposure. Many studies have investigated the effect of radiation on gonadal functions. These studies were conducted on cancer survivor patients who were exposed to pelvic radiation and to people who were exposed to radiation from atomic bombs. Long term studies investigating the effect of ionizing radiation on testis have shown that reversible azoospermia

and decreased levels of testosterone may occur after dose of 0.35 Gy, indicating that radiation affected Leydig cell function (Rowley et al. 1974). However, a radiation dose above 2 Gy could cause permanent aspermia (Ogilvy-Stuart and Shalet 1993).

Many studies have also documented the negative effects of radiation on the female reproductive system. The female reproductive system is highly susceptible to late effects of radiation exposure. Potential late effects on the female reproductive system can occur as a result of radiation exposure to the hypothalamic pituitary- ovarian axis (HPO), ovaries, and uterus (Bath et al. 2002). One of the most common late effects of radiation is ovarian failure as a result of depletion of the finite number of follicles. One study investigating the effect of total body irradiation (14 Gy) in childhood and adolescence on ovarian and uterine characteristics has shown ovarian failure, reduced uterine volume and reduced blood supply to the uterus (Bath et al. 1999). An ovarian dose of 4 Gy may cause a 30% incidence of sterility in young women, but 100% sterility in women over 40 years of age (Ogilvy-Stuart and Shalet, 1993). 14-30 Gy of radiation to the uterus is associated with reduced uterine volume, reduced myometrial elasticity, and some uterine vascular damage (Gnaneswaran et al. 2012). Radiation exposure to the uterus has also been linked to pregnancy complications such as the increased possibility for miscarriage, premature delivery, still birth, and low weight birth (Gnaneswaran et al. 2012).

Endometrial Cancer

Endometrial carcinoma, commonly referred to as uterine cancer, is the most common type of female genital tract cancer in the United States, with approximately 43,470 new cases diagnosed in the U.S in 2010, and about 7,950 deaths from the disease each year (Naumann 2011). The endometrium consists of an epithelial glandular component and a stromal component. The endometrium lines the uterine cavity and undergoes changes in response to estrogen and progesterone. Both stroma and glands proliferate under the influence of estrogen in the first half

of the menstrual cycle. In the second half of the cycle, the endometrium differentiates in preparation for implantation of an embryo under the influence of progesterone. If implantation does not occur the endometrium sloughs off and the cycle begins again. The endometrium undergoes well controlled proliferation and differentiation in premenopausal women. Endometrial carcinoma may arise when hormonal imbalance or genetic alterations affect this cycle (Di Cristofano and Ellenson 2007).

There are two different pathological types of endometrial carcinoma, type 1 and type 2. Type 1 arises in women with metabolic and hormonal disturbances such as, obesity, hyperlipidemia, diabetes, and hyper estrogenic state (Sherman et al. 1997). Type 1 carcinoma is, also called endometrioid carcinoma. It is moderate to well differentiated (low grade), confined to uterus (low stage), has favorable prognosis (85% 5-year survival rate), and is often associated with hyperplasia (Bokhman 1983). Type 2, also called serous carcinoma. It arises in women where endocrine and metabolic disturbances are absent or unclear. Type 2 are considered poorly differentiated tumors, with a deep tendency to myometrial invasion. They are estrogen independent, have doubtful prognosis (58% 5-year survival rate), and appear as atrophic lesions (Sherman et al. 1997; Bokhman 1983). Type 1 carcinoma is the most common type of the endometrial carcinoma, representing approximately 85% of cases, while serous carcinoma represents approximately 10% of cases (Di Cristofano and Ellenson, 2007).

Genetic Alterations Involved in the Development of Uterine Cancer

It has been shown that genetic alterations involved in the development of type 1 cancer differ from type 2 cancer and each one has a different gene expression profile. Type 1 shows mutations in the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), PIK3CA, K-RAS and β-catenin genes. It is also associated with microsatellite instability (MI).

While type II have loss of heterozygosity (LOH) on several chromosomes and alterations of p53 (Yeramian et al. 2012).

The tumor suppressor gene, PTEN, has been shown to be mutated in a wide variety of human tumors like brain, breast and prostate cancer and is the most frequently altered gene in endometrioid carcinoma (Li 1997). PTEN is located on chromosome 10q23. This region of the genome undergoes loss of heterozygosity in approximately 20-30% of endometrioid endometrial carcinoma (Di Cristofano and Ellenson 2007). PTEN mutation is also detected in almost of 37-61% of endometrioid endometrial carcinoma cases (Yeramian et al. 2012). In contrast to other tumor types with PTEN mutation, PTEN mutation is observed in endometrial hyperplasia and thus its mutation occurs early in the pathogenesis of endometrial cancer (Levine et al. 1998).

The PI3K/AKT Pathway

PTEN is the most frequently mutated gene in endometrial cancer and leads to activation of the PI3K/ AKT pathway and thus malignant transformation (Banno et al. 2012). The PI3K pathway is the most commonly altered pathway in the pathogenesis of endometrial cancer (Tashiro et al. 1997). The PI3K pathway transmits growth factor signals from transmembrane kinase receptors to activate cellular proliferation and survival mechanism. PI3K enzymes are categorized into three major classes: IA PI3K, IB PI3K, and class II PI3K. Class IA PI3Ks have been associated with malignant transformation in many cancer types. Class IA PI3Ks exist as heterodimers made up of a p110 catalytic and p85 regulatory subunit (Naumann 2011). Once the ligand binds to a tyrosine kinase receptor, a conformational change occurs in p-85 to release its inhibition of a p-110 catalytic site. Active p-110 converts phosphatidyl inositol 4,5 bi-phosphate to phosphatidyl inositol 3,4,5-triphosphate(PIP3). This leads to recruitment and activation of AKT proteins. PTEN acts as a negative regulator of the PI3K pathway by converting PIP3 back to PIP2, thus preventing AKT recruitment and activation. Therefore, mutation of PTEN leads to

over activation of PI3K pathway (Osaki et al. 2004). AKT, serine threonine kinase, is one of the important downstream components of the PI3K pathway. Activated AKT promotes cellular growth, proliferation, angiogenesis, and prevents apoptosis (Yamaguchi and Wang 2001). There are three isoforms of AKT proteins, AKT1, AKT2, and AKT3 and they share the same common structure among different species (Fayard 2005). The AKT proteins consist of three domains; an N-terminal domain that binds with different proteins on the plasma membrane, a central kinase domain and a C-terminal regulatory domain (Fayard 2005). Conversion of PIP2 into PIP3 leads to translocation and recruiting of AKT from the cytoplasm into the plasma membrane to bind with PIP3 via its N-terminal domain. The activation of AKT is a multistep process that requires phosphorylation of threonine 308 in the central kinase domain and phosphorylation of serine 473 within the C-terminal regulatory domain. Phospho-inositol dependent kinase (PDK1) is the enzyme responsible for phosphorylation of threonine 308, while mTORC2 (the mammalian target of rapamycin complex) is the last candidate kinase that is assumed to be responsible for phosphorylation of Serine 473 (Naumann 2011). Once activated, AKT acts as the central node of the PI3K pathway and controls multiple downstream effectors that lead to cellular growth and proliferation (figure 1).

In this study we conducted immunohistochemical staining employing a pan AKT antibody which detects all three isoforms of AKT (AKT1, AKT2, AKT3) either in phosphorylated or non-phosphorylated states. We also used a phospho-AKT antibody in our Western blot analysis. The phosphor-AKT antibody that we used in western blot detects phosphorylation sites at serine 473, which is located in the C-terminal regulatory domain of all AKT proteins. We choose to study this pathway for 2 reasons, first: as mentioned earlier most of the pathogenesis of endometrial carcinoma involves mutation in the PTEN gene and thus over-

activation of PI3K, second: radiation and stress seems to activate multiple intracellular signaling pathways including PI3K pathway and MAPK pathway (Dent et al. 2003).

The MAPK Pathway

The mitogen-activated protein kinase (MAPK) cascade is an intracellular signaling pathway that regulates different cellular activities including cell growth, cell cycle regulation, cell survival, angiogenesis, and cell migration. Like the PI3K pathway, the MAPK pathway can be activated when a ligand binds with a tyrosine kinase receptor on the cell surface or other receptors like G-protein coupled receptor and cytokine receptors (Friday and Adjei 2008).

The MAPK pathway includes several signaling molecules such as RAS, RAF, MEK, and ERK. The activation of this pathway begins when a mitogen binds and activates cell membrane receptor tyrosine kinase causing a downstream signaling cascade. Activated tyrosine kinase leads to activation of GTPase RAS protein that resides in the plasma membrane and acts as an upstream switch for activation of other downstream effectors in the MAPK pathway. Activated RAS leads to recruitment and activation of serine threonine RAF kinases. Active RAF proteins in turn phosphorylate and activate MEK1 and MEK2. MEK1 and MEK2 are serine/threonine and tyrosine kinases that activate and phosphorylate extracellular signal regulated kinase (ERK1) and ERK2. ERK proteins have numerous downstream effectors which regulate cell growth and proliferation (McCain 2013). Oncogenic alterations in the RAS/MAPK pathway are found in endometrial cancer in the form of activating KRAS mutations (Duggan et al. 1994). The mutation of KRAS has been found in 10-30% of endometrial carcinomas (Dobrzycka et al. 2009).

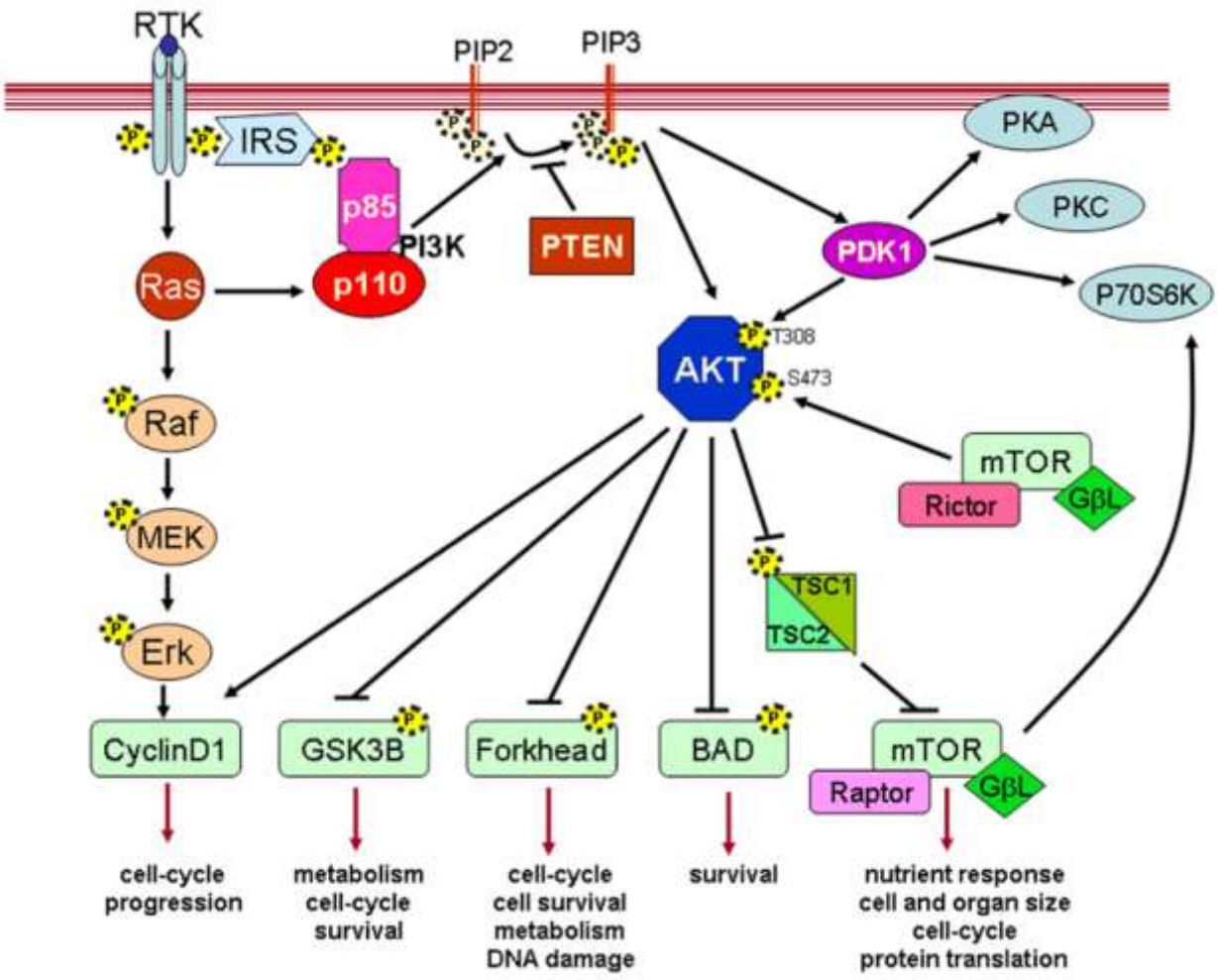


Figure 1: Overview of The PI3K/AKT and MAPK pathways (Carnero and Paramio 2014).

Objectives and Significance of this Study

There were two major aims of this study:

Specific aim 1: To determine the effect of high radiation exposure and simulated spaceflight conditions on the incidence of uterine cancer.

Specific aim 2: If cancer is detected, to identify the cell type affected.

Uterine cancer is the fourth most common cancer in women in the United States and the most commonly diagnosed gynecologic cancer. In 2013, 50,560 women in the United States

were diagnosed with uterine cancer. 9,325 women in the United States died from uterine cancer in that year (U.S. Cancer Statistics Working Group 2017). In the United States roughly 1 in 530 people between the ages of 20-39 is a childhood cancer survivor (CureSearch for Children's Cancer 2018). The unique health related issues faced by these cancer survivors has not been adequately studied. Irradiation used in the treatment of childhood cancers has been shown to have long term effects on female reproductive structures, especially the ovary and the uterus. Exposure to radiation therapy in childhood has impaired uterine function, causing reduced uterine volume, decreased myometrial elasticity, and some uterine vascular damage (Gnaneswaran et al. 2012). However, the effect of radiation directly on the reproductive tissue has not been examined. Disruption to normal reproductive functions are of great public health concern as they increase the health issues in the mother and cause higher rates of mortality in the offspring. The space flight environment is also high in radiation. As humans continue venturing deeper and longer into space, they will be exposed to a high radiation environment. This is expected to cause effects similar to those seen in clinical irradiation. Our study addressed the changes associated with radiation and microgravity in female reproductive tissues, especially the increased risk for uterine cancer.

CHAPTER 2

MATERIALS AND METHODS

All tissues utilized in this study came from the laboratories of Drs. Xiao Mao and Mike Pecaut of Loma Linda University in Loma-Linda, California. Six month old C57 BL/6 female mice were maintained 4/cage under a constant ambient temperature of 65.8°F with a 12h light/dark cycle. After 1 week of acclimatization, the animals were randomized into 4 groups (6/group) as follows: 1) age-matched controls, 2) low-dose-rate (LDR) γ-irradiation, 3) unloading with tail suspension, and 4) LDR + tail suspension. Cobalt^{57} (57CO) plates (0.04Gy at 0.01cGy/h) were used to deliver irradiation to the whole body of 6-month-old adult C57 BL/6 mice (n=4-6/group) to simulate the radiation of spaceflight. Anti-orthostatic tail suspension, also known as HU, was used to model the unloading, fluid shift, and physiological stress aspects of spaceflight. Mice were hindlimb suspended and/or irradiated for 21 days to test the potential interaction. In that 21-day unloading period, the mice were individually housed. Mice were euthanized with 100% CO_2. Mice were euthanized either 1 month, 4 months, or 9 months following completion of treatment (radiation, HU, or radiation combined with HU). Vaginal smears were performed on each animal at the time of euthanasia to allow estrous cycle stage determination. Uterine tissues were harvested by a laboratory technician within 30-60 minutes of euthanasia. Following excision, the uteri were separated into left and right portions. One portion was placed in RNAlater and the other portion was preserved in 4% paraformaldehyde. The tissues were then sent to the laboratory of Dr. Allan Forsman at East Tennessee State University. Upon arriving, paraformaldehyde preserved tissues were trimmed of fat and stored in 70% ethanol (ETOH). Tissues stored in RNAlater were placed in -80° C storage for later analysis.

Uterine tissues stored in RNAlater were divided into two sections. One section was used in Western blot analysis while the other section was returned to the RNAlater for future analysis.

Western Blot Procedure

Uterine tissues were homogenized in 280 µl of RIPA buffer (150 mM NACL, .1% triton x-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 Mm TrisHCL [PH 8.0]) with 100 µl protease inhibitor cocktail (category # B14001), 100 µl phosphatase inhibitor cocktail (category # B15001-B) and 100 µl phosphatase inhibitor cocktail (category # B15001-A). Homogenate was centrifuged for 10 min at 16,000 RPM (Revolutions Per Minute) and placed on ice. The supernatant was then aspirated and placed in a fresh microfuge tube kept on ice. Proteins were quantified using the Bradford assay. The Bradford protein assay depends on using standard proteins of known concentration to create standard curves and equations that will be used to estimate the concentration of protein of unknown solutions. This method is based on the proportional binding of Coomassie blue dye to proteins. When there are more protein contents in the solution, more Coomassie dye will bind with protein and create a significant change in color of the mixture. The Bradford assay uses Coomassie brilliant blue G250 which has a red brown color in acidic solution, but when proteins bind with this dye the color turns blue. The protein-bound form of the dye has an absorption spectrum maximum at 595 nm. The increase of the absorbance at 595 nm is positively correlated with the amount of the bound dye and with the amount of protein in the solution. A spectrophotometer was used to measure the light intensity of light being absorbed or reflected and express these intensities in the form of absorbance values. For this assay we used the dye reagent and protein assay standards which are set at seven prediluted concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/µl). The first step in protein quantification is dilution of protein samples with distilled water then vortex. This step was necessary because homogenization was done in RIPA buffer and RIPA buffer interferes with the protein-dye interaction. Because of this interference the protein samples should undergo a 40 – 50 X dilution before beginning the protein estimation. To accomplish this, 245 µl of

distilled water was added to 5 µl protein samples. Then 30 µl of diluted protein samples and standards were added to the wells of assay plate. For accuracy this last step was performed in triplicate. For each well containing the samples and the standards, 270 µl of Coomassie plus (pro # 1856209) was added and incubated for 10 minutes at room temperature. A spectrophotometer was used to read the plate and obtain the readings of standard protein concentration versus the absorbance. A standard curve calculated from the readings of the standards was used to predict the concentration for our protein samples.

To prepare samples for western blotting, the sample tubes were placed in a hot plate at 100℃ for 5 minutes, placed on ice for 5 minutes, then vortex and spun. An equal amount of protein was loaded into the wells of TGx pre-cast gels (category # 456-1086, lot # 64074007). 5 µl of ladder was added to the first lane (category # 26616) and 15 µl of each sample was added to the remaining lanes. The container was filled with running buffer and the gel was run at 300 volts for 25 minutes. Protein was transferred to an Immobilon polyvinylidene fluoride (PVDF) membrane over 1 hour using 100 V. After 1 hour the membrane was stained with ponceau red to check the transfer quality. The membranes were then washed with TBS-T 3x for 5 minutes, then blocked in 10 ml western blot blocking (TBS, 2% bovine serum albumin, 2% fetal calf serum, 0.1% sodium azide) for 1 hour. Membranes were exposed to a 1:1000 primary antibodies (10 ml of TBST, 10 µl of phospho AKT, and 10 µl of 20% sodium azide) of rabbit anti- mouse phospho AKT (cat # 4051). Membranes were washed with TBS-T three times for five minutes. Then membranes were incubated with appropriate 1:10000secondary antibodies (2.5 µl of goat anti rabbit #31460, 0.5 g of non-fat milk, 25 ml of TBST) coupled to horse radish peroxidase (HRP) for 1 hour at room temperature. After washing the membranes three times for 5 minutes, signals were visualized using chemiluminescent lumigen (100 µl of reagent A, 100 µl of reagent B and 1

ml of distilled water). Then we used alpha view software to read the band intensity of the target proteins.

Membranes were stripped using mild stripping buffer for 10 minutes, PBS was added two times for 10 minutes and poured off, then TBS-T was added two times for 5 minutes and poured off. Membranes were blocked again with western blot blocking buffer. Membranes were then ready for application of other primary antibodies such as rabbit anti- total AKT (468S), rabbit phospho mapK 42/44 (Cat # 4370), total 42/44 ERK, and β-tubulin. β-tubulin was used as a housekeeping protein to do normalization with target proteins. Membranes were incubated for 1 hour with appropriate secondary antibody (goat anti mouse #7076s or goat anti rabbit #31460).

Histological Examination

Paraformaldehyde preserved uterine horns were divided into proximal, middle, and distal portions. The middle portion was embedded in paraffin for use in this study. The first step in tissue processing is tissue dehydration. Because paraffin is immiscible in water, tissue dehydration is required to remove water from tissues before paraffin infiltration is performed.

Paraffin embedding is necessary to render the tissue firm enough to be sectioned on a microtome. In order to infiltrate the tissue with paraffin the water/fluid component of the cell must be replace with melted paraffin. This is accomplished by immersing the tissue in increasing concentrations of ETOH until the tissue is infiltrated with 100% ETOH. To accomplish this infiltration the tissue is removed from the 70% ETOH in which it was stored and placed on 80% ETOH for 30 minutes. The tissue is then moved to 90%, ETOH, 95% ETOH, 95% ETOH, and finally 100% ETOH for 30 minutes each, with the 100% stage done twice. This gradual dehydration prevents distortion and damage of the cells/tissue. Because ETOH is not miscible with paraffin, further steps must be taken to replace the ETOH with melted paraffin. This is accomplished by immersing the tissue in a 50:50 mixture of 100% ETOH and a dehydrating

agent known as Citrisolv for 30 minutes. It should be noted that Citrisolv is miscible with both ETOH and paraffin. Following this 30-minutes immersion the tissue is place in 100% Citrisolv for 30 minutes followed by immersion in a 50:50 mixture of Citrisolv and melted paraffin. This stage, and all subsequent stages must be carried out at 60-65°C to insure the paraffin remains liquid. The tissue is then immersed in 100% paraffin for 1 hour followed by another 1-hour immersion in another container of 100% paraffin. Following the second hour of 100% paraffin immersion the paraffin is placed in an embedding mold that has been treated with mold release. Mold release is made of a solution consisting of 5 ml of glycerin and 400 ml of 75% ETOH. Embedding molds are treated by immersion in mold release and then allowing to dry and then repeating this step. The remaining steps in the embedding procedure are carried out on a tissue-Tek embedding console. One the tissue is place in the embedding mold a small amount of melted paraffin is dispensed into the embedding mold and the tissue is centered in the embedding mold. Once the tissue is in the desired position the embedding mold is moved to the cooling plate of the console and an embedding ring is added to the top of the embedding mold. Melted paraffin is then added to the embedding ring until the ring is filled. The embedding mold/ring assembly is then moved to the final cooling area of the embedding console and the paraffin allowed to cool/solidify. Once solidified the tissue/block can be stored indefinitely.

Embedded tissues were then sectioned at 4μm and placed on glass microscope slides. Slides were then stained using a standard H&E staining technique. The slides were examined microscopically for visible signs of cancer.

For immunohistochemical (IHC) staining we prepared three microscopic slides for each specimen. Two slides had the primary and secondary antibodies, while the third slide had negative control, or had primary or secondary antibody only. The primary antibody used in this procedure was a pan-AKT antibody (cat#8805). IHC staining was used to identify the presence

and location of AKT in these tissues. The first step in IHC was deparaffinizing and rehydrating the tissue sections using xylene and series of alcohol concentrations. Paraffin should be removed to expose the epitope and allow its binding with primary antibody. Also, failure to remove paraffin will trap excess chromogen causing background staining. Rehydration will be important to allow the target antigen to interact with antibodies. Deparaffinization is accomplished by immersing microscopic slides twice in xylene for 3 minutes each. Then slides are immersed in series of alcohol concentrations for rehydration. The slides immersed in 100% ETOH, 95% ETOH, 80% ETOH, and 70% ETOH for 3 minutes each with 100% stage done twice. Finally, tissues were immersed in distilled water 3 times for 3 minutes each.

Microscope slides were then placed in a container filled with tap water and transferred to an autoclave for 15 minutes at 122°C of wet cycle. This step was done to retrieve and unmask the antigen binding sites that were crosslinked during the paraformaldehyde fixation. Following antigen retrieval 3% hydrogen peroxide was applied to each slide to prevent endogenous peroxidase activity and therefore prevent nonspecific binding and false positive detection. This blocking step was performed at room temperature for 45 minutes. Slides were then rinsed with 1-X rinse buffer (LOT# 2834466). To prepare 100 ml of 1X rinse buffer we added 5 ml of 20X rinse buffer from the IHC kit, 95 ml of distilled water, and added 100 µl of tween 20. To prevent nonspecific binding of primary and secondary antibodies with non- antigen proteins, we applied 2 drops of blocking reagent to the specimen and incubated at room temperature for 45 minutes in an enclosed container. The slides were then rinsed with 1X rinse buffer. Two types of blocking solution were used during the procedure, blocking reagent in IHC kit (LOT# 2834466) which was later replaced with carrier solution (1 ml of normal goat serum, 1.5 grams of bovine serum albumin, 1.5ml of 20% triton X-100, and 97.5 ml of PBS). Normal serum carries antibodies that will bind to the non-specific epitopes in the sample, thus preventing non-specific binding with

primary antibody. A serum identical to the host animal of the secondary antibody or from an unrelated species is recommended to prevent interactions with primary or secondary antibodies, or with the tissues/cells being stained.

Two drops of blocking solution were added per slide and the slides subsequently incubated for 45 minutes at room temperature. The next step was rinsing and applying primary antibody 1: 1000 (1 µl of p-AKT cat # 8805, 998.5 µl of PBS, and 0.5 µl of tween 20). Primary antibody was applied at 4°C overnight. The slides were then rinsed with 1X rinse buffer. Secondary antibody was applied for 45 minutes at room temperature. The antigen bound antibody was then localized by adding streptavidin HRP (two drops for 45 minutes) which generates amplified signal when chromogen reagent is added. The IHC kit we used contained two bottles of chromogen reagent, DAB chromogen A and B. We mixed chromogen A and B in a 1:25 ratio and applied to the slides for ten minutes then rinsed. Hematoxylin counterstain was applied for 1 minute, rinsed, and placed in container filled with tap water. The counterstain provides contrast to primary stain and helps to pin point the exact position of positive staining cells. The last step in IHC was sealing the sample and prevent enzymatic produced solubilization by dehydration through a graded series of alcohol and xylene based mounting media (paramount) followed by cover slipping. Dehydration is accomplished by immersing slides in 70% ETOH, 80% ETOH, 95% ETOH, 100% ETOH for two minutes each with the 100% ETOH stage done twice. Then slides are immersed twice in citrisolv for 3 minutes each and then cover slipped.

To evaluate the IHC results, the slides were scored in triplicate (each slide was read three times by the same observer) using the combined location and intensity scoring system, where a score of 0 indicates no staining reaction or weak focal reaction, a score of 1 for intense focal or mild diffuse reaction, a score of 2 for moderate diffuse reaction, and a 3 for intense diffuse reaction (Fedchenko and Reifenrath 2014).

Statistical Analysis

Relative band densities of western blots were calculated for each protein of interest using alpha view software. These were normalized to β-tubulin controls for graphical depiction of western blot results. Graph Pad Prism 7 was used for the statistical analysis and generation of graphs. Data were summarized by the mean and standard deviation. Group mean responses were compared by one-way analysis of variance (ANOVA) where p-values of 0.05 or smaller were considered significant.

CHAPTER 3

RESULTS

Gross Morphology of H&E Stained Uterine Tissues

To investigate possible uterine morphological and structural changes induced by simulated microgravity, whole body radiation, or the two-combined, hematoxylin and eosin (H&E) staining of the uterine tissues was performed. The gross morphology of the H&E stained tissues was microscopically evaluated. Represented examples of these stained tissue can be seen in figure 2. Results of the H&E staining revealed no differences between these samples at the gross morphological level. No indication of abnormal sizes or shapes of cells or abnormal growth was noted.

Figure 2A

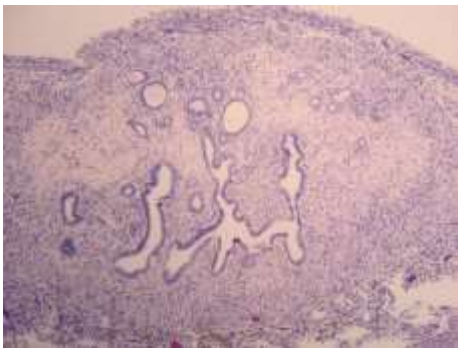


Figure 2B

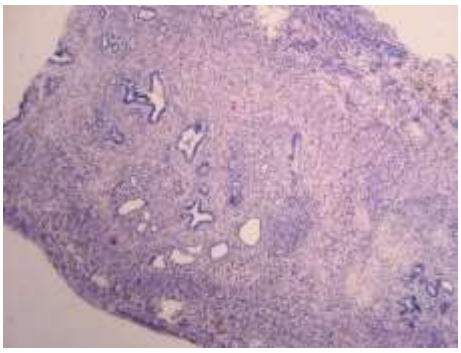


Figure 2C

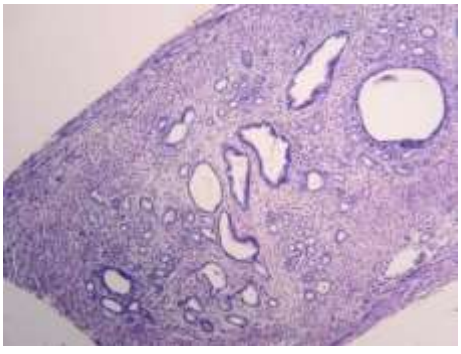


Figure 2D

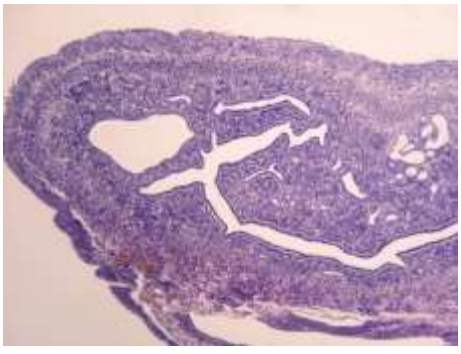


Figure 2: Longitudinal section of H&E stained uterine tissues from the animals sacrificed 9-months post treatment (X100) for A) control mouse, B) radiated mouse, C) unloaded mouse, and D) radiated and unloaded mouse.

Immunohistochemical Evaluation

To investigate the possible cancer-causing effects of whole body radiation, HU, or a combination of the two on uterine tissues, we used immunohistochemical staining (IHC) using a pan AKT antibody. The IHC staining results show that uterine tissues from animals sacrificed 4 months post treatment had mild diffuse expression of AKT. There was no difference between the control and other treatment groups as seen in figure 3.

Figure 3A

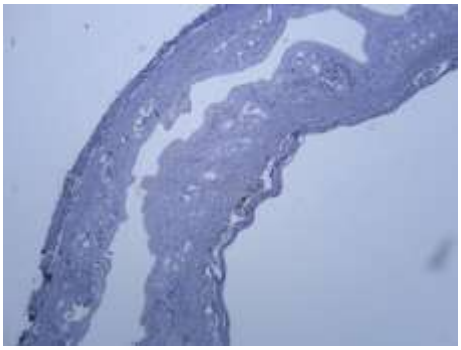


Figure 3B

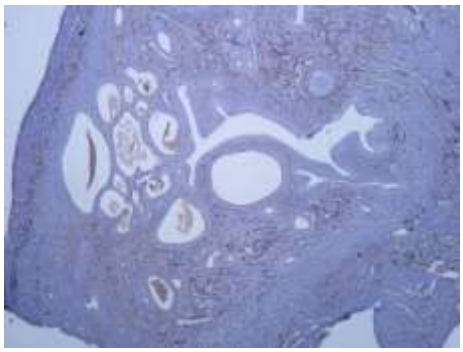


Figure 3C

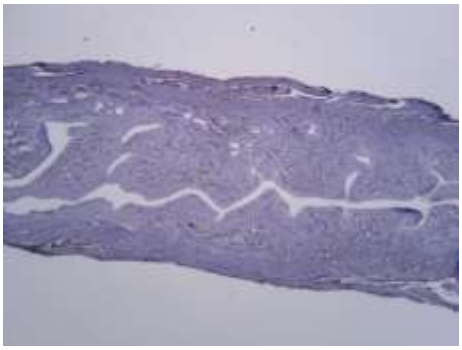


Figure 3D

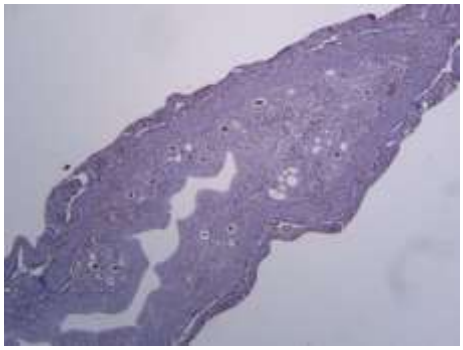


Figure 3: Longitudinal section of IHC stained uterine tissues from the animals sacrificed 4-months post treatment (X100) for A) control mouse, B) radiated mouse, C) unloaded mouse, and D) radiated and unloaded mouse.

There is no difference in AKT expression level between the various treatment groups in the tissues from the animals sacrificed 9 months post treatment. However, the positive staining reaction was moderate to intense diffuse compared with the various treatment groups in the

tissues from the animals sacrificed 4 months post treatment as shown in figure 4. IHC staining results also show that AKT expression was visible in both the endometrial and myometrial layers of the uterus as shown in figure 4E.

Figure 4A

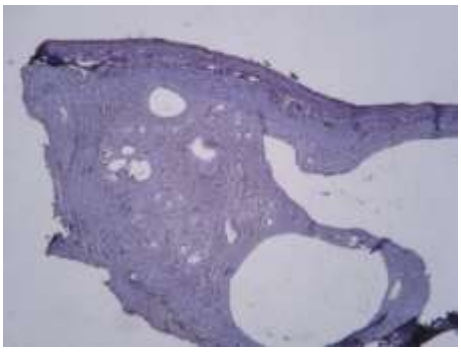


Figure 4B

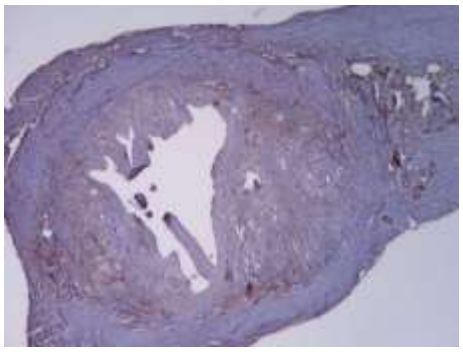


Figure 4C

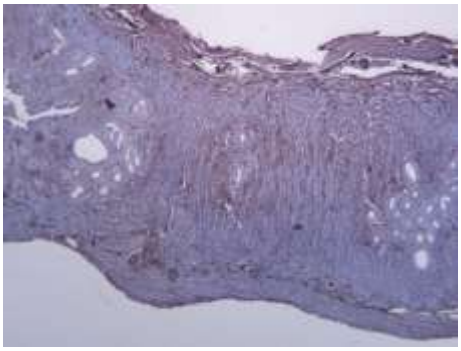


Figure 4D

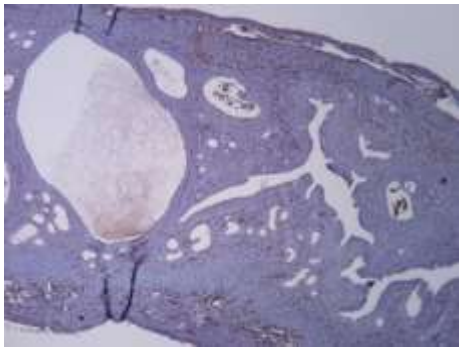


Figure 4E

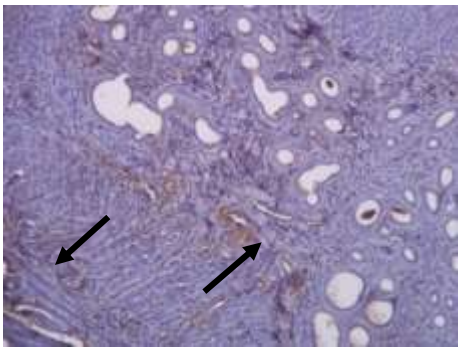


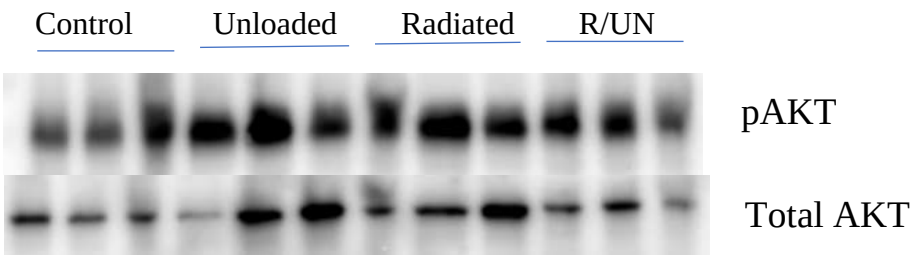
Figure 4: Longitudinal section of IHC stained uterine tissues from the animals sacrificed 9-months post treatment (X100) for A) control mouse, B) radiated mouse, C) unloaded mouse, D) radiated and unloaded mouse, E) and R/UN section show AKT positive staining in both endometrial and myometrial layers.

Western Blot Analysis

Expression of pAKT in 4 Months and 9 Months Post Treatment Uterine Tissues

To investigate whether or not whole-body radiation, HU, or the combination of the two causes over activation of the PI3K pathway, western blot analysis was performed to identify the level of pAKT proteins in 4 months and 9 months post treatment samples. There was no significant difference of pAKT protein level normalized data between control, HU, radiated, and radiated and unloaded groups in both 4 months and 9 months post treatment samples as seen in figures 5 and 6.

A



B

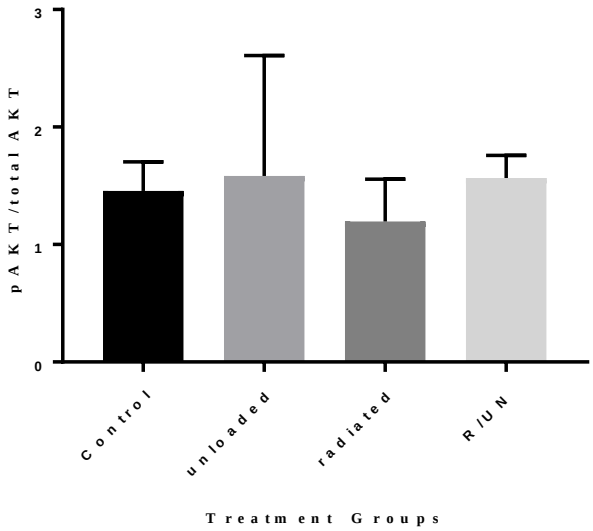
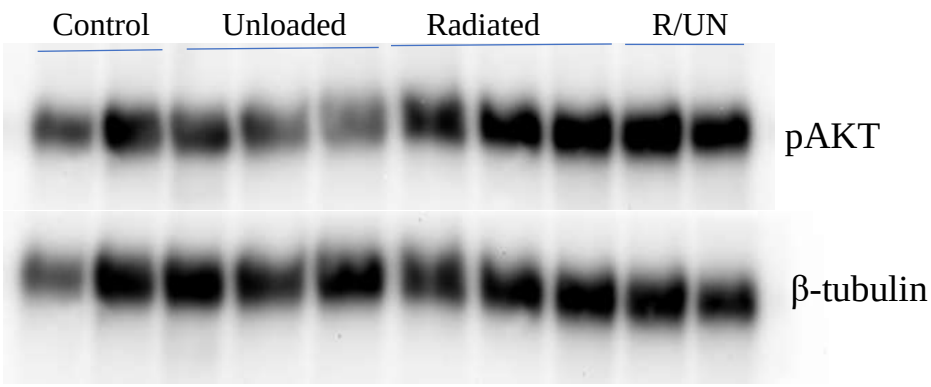


Figure 5: pAKT content in uterine samples from animals sacrificed 9 months post treatment. A: Representative Western blot of Uterine pAKT protein; B: Densitometry of the ratio of pAKT bands to total AKT. No statistical difference between groups (p = 0.82). Data was expressed as the mean ± SD (n = 3 mice/group).

A



B

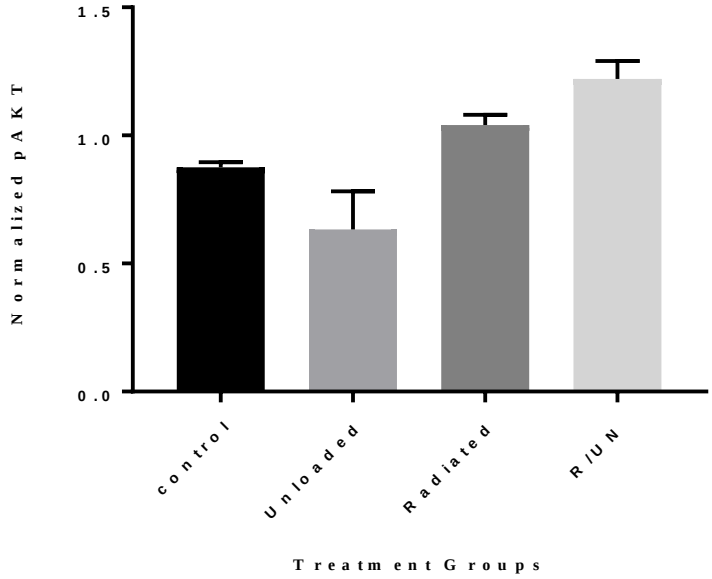
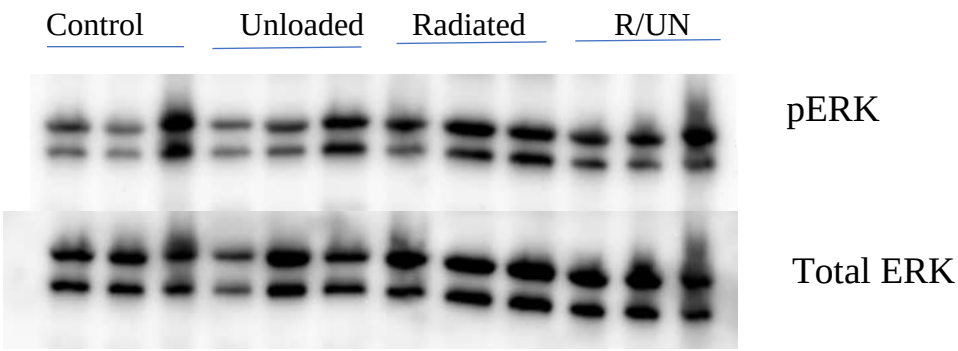


Figure 6: pAKT content in uterine samples from the animals sacrificed 4 months post treatment. A: Representative western blot of Uterine pAKT protein level; B: Densitometry of pAKT bands normalized to β-tubulin. No statistical difference between groups (p = 0.11). Data are expressed as the mean ± SD (n = 2-3 mice/group).

Expression of pMAPK in 4 Months and 9 Months Post Treatment Uterine Tissues

To investigate whether or not radiation, HU, or a combination of the two over activate the MAPK pathway, western blot analysis was performed to identify the level of phospho ERK and total ERK from the animals sacrificed 4 months and 9 months post treatment. Western blot analysis revealed no significant difference of phospho ERK normalized data among control, unloaded, radiated, and radiated and unloaded groups ($p>0.05$) as seen in figures 7 and 8.

A



B

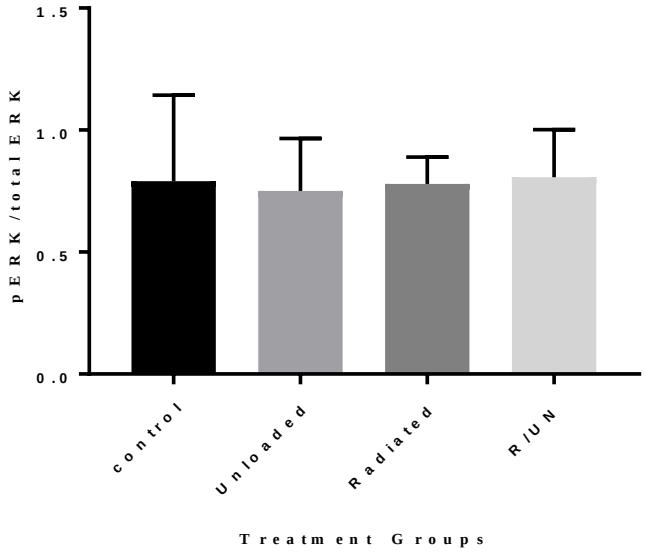
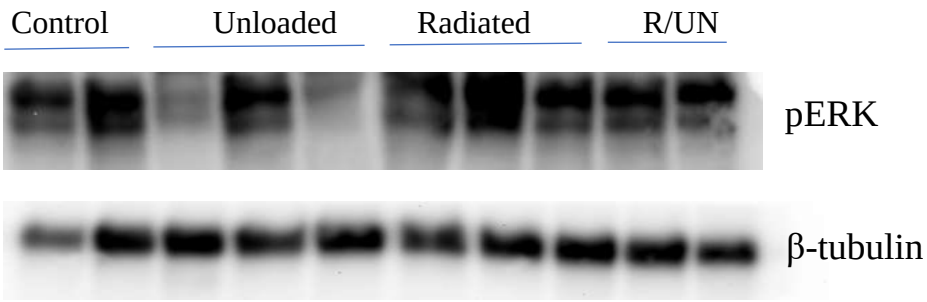


Figure 7: pERK content in uterine samples from the animals sacrificed 9 months poste treatment A: Representative Western blot of Uterine pERK protein level; B: Densitometry of the ratio of pERK bands to total ERK. No statistical difference between groups (p =0.99). Data was expressed as the mean ± SD (n = 3 mice/group).

A



B

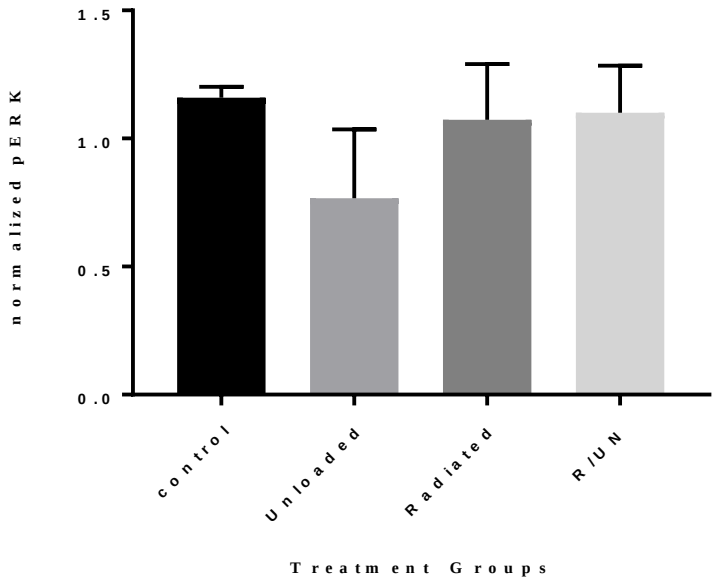


Figure 8: pERK content in uterine samples from the animals sacrificed 4 months poste treatment. A: Representative Western blot of Uterine pERK protein level; B: Densitometry of pERK bands normalized to β tubulin. No statistical difference between groups (p = 0.34). Data was expressed as the mean ± SD (n = 2-3 mice/group).

CHAPTER 4

DISCUSSION AND CONCLUSIONS

This study was designed to investigate whether radiation, simulated microgravity or both may increase the incidence of uterine cancer, and if so, which portion/s of the uterus, endometrium or myometrium, are more vulnerable. Experimental studies using animal models and epidemiological studies of atom bomb survivors and survivors of nuclear accidents have shown radiation to be a universal carcinogen (Little 2000). The carcinogenic effect of radiation comes from radiation's ability to penetrate protective cellular barriers and deposit energy inside cells. Radiation is known to cause cancer by one of three mechanisms: alteration in the structure of genes or chromosomes, alteration in gene expression, or through oncogene viruses (Beir 1990).

PI3K and MAPK pathways are common intracellular pathways that have been shown to be activated in uterine cancer (Yeramian et al. 2012). The PI3K pathway activation begins when a ligand binds to tyrosine receptor kinase causing PI3K enzyme to convert PIP2 into PIP3. This leads to recruitment of a cytosolic inactive form of AKT to the plasma membrane where it binds with PIP3. AKT binding with PIP3 induces conformational changes of AKT which enables its activation by various kinases in the plasma membrane. PDK1 enzyme phosphorylates AKT at threonine residue, while mTORC2 directly phosphorylates the serine residue of AKT. This double phosphorylation fully activates AKT. AKT is one of the major downstream effectors of the PI3K pathway. Activated AKT in turn activates other effectors important for cell growth and proliferation. The PTEN gene, which is highly mutated in uterine cancer, acts as negative regulator of the PI3K pathway by conversion PIP3 back into PIP2 which prevents AKT recruitment and activation.

In this study, pAKT levels were measured using western blot analysis. No significant difference in normalized level of pAKT to β-tubulin or in relative level of pAKT to total AKT was noted in the different treatment groups utilized. The treatment groups utilized included animals sacrificed 4 months and 9 months after total body radiation, HU or both.

The MAPK pathway has been shown to be activated under the effect of radiation and stress conditions. Also, the MAPK pathway has been found to be upregulated in 10-30% of endometrial cancer. Like the PI3K pathway, activation of the MAPK pathway begins when a ligand binds to the membrane tyrosine kinase receptor, causing a downstream signaling cascade. pERK is one of the downstream effectors of the MAPK pathway. Phosphorylated ERK activates other proteins, which have an important role in cell growth and survival.

In this study, the pERK level was measured using western blot analysis. No significant difference in pERK normalized to β-tubulin level or relative to total ERK were noted in the different treatment groups utilized. The treatment groups utilized included animals sacrificed 4 months and 9 months after total body radiation, HU or both.

The western blot analysis results were consistent with hematoxylin and eosin (H&E) stained uterine tissues. H&E staining of uterine tissues was performed to detect any possible abnormal changes at the gross morphological level under the effect of whole body radiation, microgravity, or both. The results of H&E stained uterine tissues show no morphological differences between the treatment groups in animals sacrificed 4 months and 9 months after total body radiation, HU or both.

An IHC staining technique was also used to determine the expression level of AKT proteins in uterine tissues. Pan AKT primary antibody was used in the IHC staining technique. Pan AKT antibody detects the AKT proteins either in phosphorylated or non-phosphorylated

states. The results of IHC show mild diffuse staining of pan AKT in uterine tissues. No difference between treatment groups of animals sacrificed 4 months after total body radiation, HU or both was detected. IHC staining results for uterine tissues from animals sacrificed 9 months after whole body radiation, unloading, or both show more intense staining of pan AKT compared to 4 months treatment groups.

These results indicate that small dose 0.04 Gy (40 milliseivert) whole body gamma irradiation, HU, or both dose not induce the formation of uterine cancer formation. The fact that ionizing radiation causes cancer in humans has been documented. By 1902, the first radiation induced cancer was reported as skin cancer and the first report of radiation induced leukemia occurring in five radiation workers appeared in 1911 (Little 2000). These findings of radiation carcinogenesis were further supported by experimental studies of animal models and by long term follow up studies of atomic bomb survivors. These studies also documented the fact that radiation is a relatively weak carcinogen and mutagen compared with other chemicals, but its effect can be modulated significantly by other secondary factors (Beir 1990).

Carcinogenesis is a multistep process which involves interaction of many external (chemical and physical) and endogenous factors such as age, sex, genetic background, and immunologic status (Beir 1990). The multistage model of carcinogenesis indicates that cancer formation requires 3 steps: initiation, promotion, and progression.

The initiation process is irreversible. The radiation is considered an initiator of cancer. The ability of radiation to initiate cancer could be attributed to the wide spectrum of DNA lesions. It is well documented that the main target of radiation is DNA, which is damaged by the radiation. Radiation causes a wide spectrum of DNA lesions including damage to nucleotides, SSBs, and DSBs. DSBs are considered the primary lesion in the induction of gene mutation and

chromosomal aberrations (Mullenders et al. 2009). DSBs are repaired mainly via error prone NHEJ.

The second process of carcinogenesis is promotion. This stage is reversible and requires stimulation of cell division. Ionizing radiation could be a promotor if given at high enough dose. Whereas the initiating agents, radiation, are carcinogenic by themselves if they were given in a large dose, promoting agents must be given repeatedly to over a long period of time, during which successive promotion may occur (Beir 1990; Little 2000).

Progression is the third stage of carcinogenesis. This stage occurs when additional genetic alterations allow the cell to be invasive and metastatic. Ionizing radiation could progress cancer formation due to its ability to deactivate tumor suppressor genes. This multistage nature of carcinogenesis indicates that radiation is a contributing factor and by its interaction with various secondary factors can lead to cancer progression.

Most studies investigating the relationship between radiation and cancer risk have looked at people who were exposed to high levels of radiation. It is more difficult to measure the risk of cancer after exposure to lower doses of radiation. Thus, whether low dose of ionizing radiation LDIR is a risk factor for cancer remains controversial (Mullenders 2009). A study done by Zhang et al. (2015) using a mouse model has revealed that a risk of mammary cancer from low dose radiation depends greatly on the genetic makeup of these mice. According to this study, cancer susceptibility is a complex process that depends on interaction between multiple factors, such as exposure to low dose radiation, genetic background, and the tumor microenvironment (immune, endocrine, and vascular system). They identified 13 genetic loci which contribute to tumor susceptibility when a mouse is exposed to radiation. Further research is needed to identify how these genetic loci affect cancer development. According to Zhang's study, cancer risk from

exposure to low dose radiation differs among individuals. Therefore, one individual may have a higher risk to cancer after radiation exposure than another individual according to their genetic makeup.

AKT, serine/threonine kinase, is considered one of the most critical regulators of cell cycle control, apoptosis, and metabolism. AKT is central to many human physiological processes, but an increase of function of AKT is linked to multiple diseases. An example of pathological involvement of PI3K pathway is a study done by Suman et al. (2012) investigated the effect of radiation exposure on mammary tissues. The researchers exposed mice to 2 Gy whole body gamma radiation. The results showed increase incidence of breast cancer. According to this study, radiation lead to increase expression of estrogen which binds to estrogen receptor alpha (ERα) resulting in activation of PI3K pathway. Western blot analysis of the uterine tissues in our study shows a clear band for pAKT in all treatment groups without significant differences. AKT plays a physiological role in uterine tissue regulating factors related to cell death and proliferation, such as embryo implantation, decidualization, and menstruation (Fabi and Asselin 2014).

ERK proteins play a significant role not only in pathological processes but also in different physiological processes such as proliferation, differentiation and development (Shaul and Seger 2007). The physiological role of the PI3K and MAPK pathways in the uterus involve functions related to tissues remodeling that occur during the menstrual cycle and during embryo implantation. Cell migration is an example of physiological processes used by cells during several processes such as wound healing. The endometrium is plastic tissue which undergoes adaptive reactions to the physiological changes that occur in different phases of the menstrual/estrous cycle and during embryo implantation (Gentilini et al. 2007). When endometrial tissue loss occurs during menstruation, endometrial cell growth, angiogenesis, and

cell migration act together to reestablish organ integrity and refill the space created by tissue loss. According to the study of Gentilini et al. (2007) MAPK and PI3K pathways are activated in response to growth factors and cytokines and have important role in cell migration and growth during endometrial remodeling. These proteins are expressed normally in uterine tissues in order to perform necessary physiological functions. This could explain the visible bands of pERK and pAKT in the western blots of our uterine tissues.

IHC staining results showed more intense staining for AKT proteins in animals sacrificed 9 months post treatment compared to animals sacrificed 4 months post whole body radiation, microgravity, or both. This may indicate higher level of AKT proteins in 9 months samples compared to 4 months samples. Animals sacrificed 9 months post whole body irradiation, microgravity, or both may live enough time to show cellular changes after treatment. This may indicate higher cellular activity in 9 months treatment groups which could be indicative for abnormal changes or hyperplasia in uterine tissues. Further research using more quantitative methods is needed to explain this increase.

Linear energy transfer (LET) is the energy transferred per unit length of track. Low LET radiation travel longer distance through matter and deposits less energy. High LET radiation deposits large amount of radiation in a small distance. So, high LET radiation causes more cellular damage than low LET radiation (Cucinotta and Durante 2006). Spaceflight radiation is considered high LET, while X-rays and gamma rays are low LET radiation. The main health concerns of astronaut's health are exposure to space radiation. Space radiation includes high charge and energy protons (HZE) and secondary radiation produced by nuclear reaction in space craft walls or in tissues. The possible mission to mars could result in whole body radiation dose of about 1 Sievert or more.

Studies have shown that high LET radiation produces different DNA lesions and complex breaks compared with low LET radiation (Cucinotta and Durante 2006). Complex damage occurs within a localized region of DNA making the repair more difficult. This will cause more complex chromosomal rearrangement which may lead to cancer formation or cell death. Being limited in its ability to accurately simulate the radiation component of spaceflight and therefore, although we did not see any incidence of uterine cancer, this would not preclude astronauts exposed to higher energy radiation from developing uterine cancer. Some future aims in working on this project include using higher level of radiation dose and studying the effect of radiation, microgravity, or both directly on the PTEN and KRAS genes.

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