



Review article

Biological effects of space radiation and development of effective countermeasures



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ABSTRACT

As part of a program to assess the adverse biological effects expected from astronauts' exposure to space radiation, numerous different biological effects relating to astronauts' health have been evaluated. There has been major focus recently on the assessment of risks related to exposure to solar particle event (SPE) radiation. The effects related to various types of space radiation exposure that have been evaluated are: gene expression changes (primarily associated with programmed cell death and extracellular matrix (ECM) remodeling), oxidative stress, gastrointestinal tract bacterial translocation and immune system activation, peripheral hematopoietic cell counts, emesis, blood coagulation, skin, behavior/fatigue (including social exploration, submaximal exercise treadmill and spontaneous locomotor activity), heart functions, alterations in biological endpoints related to astronauts' vision problems (lumbar puncture/intracranial pressure, ocular ultrasound and histopathology studies), and survival, as well as long-term effects such as cancer and cataract development. A number of different countermeasures have been identified that can potentially mitigate or prevent the adverse biological effects resulting from exposure to space radiation.

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Abbreviations: ARS, acute radiation sickness; aPTT, activated partial thromboplastin time; BBI, Bowman–Birk inhibitor; BBIC, BBI concentrate; BFO, blood forming organs; BK, bradykinin; CNS, central nerve system; CT, computed tomography; DCF, dichlorofluorescein; DIC, disseminated intravascular coagulation; DMF, dose modifying factor; DNA-PKcs, DNA-dependent protein kinases; DTH, delayed type hypersensitivity; ECM, extracellular matrix; EGb76, quercetin; eSPE, simulated electron SPE; EVA, extra-vehicular activity; GI, gastrointestinal; GCR, galactic cosmic rays; G-CSF, granulocyte colony-stimulating factor; HDR, high dose rate; HS, hindlimb suspension; HZE particles, highly energetic, heavy, charged particles; ICRP, International Commission of Radiation Protection; IFN- α , interferon-alpha; INR, the patient's 'test' PT value divided by the laboratory 'normal' PT value, raised to the power of the International Sensitivity Index; ISS, International Space Station; LAD, left anterior descending; LBP, lipopolysaccharide binding protein; LD₅₀, dose expected to kill 50% of the treated subjects; LDR, low dose rate; LET, linear energy transfer; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; NAC, N-acetyl cysteine; NASA, National Aeronautics and Space Administration; NCRP, National Council on Radiation Protection and Measurements; NK, Natural Killer; PAMP, pathogen associated molecular patterns; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PHA, phytohemagglutinin; PMN, polymorphonuclear leukocyte; pSPE, simulated proton SPE; PT, prothrombin time; PWS, partial weight suspension; RBE, relative biological effectiveness; SCR, solar cosmic radiation; SEB, surrogate endpoint biomarker; SeM, L-selenomethionine; SOBP, spread out Bragg peak; SPE, solar particle event; SWT, Si–Wu–Tang; TAS, total antioxidant status; TBI, total body irradiation; TF, tissue factor; vWF, von Willenbrand factor; WBC, white blood cell.

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1. Introduction

As reviewed by Hellweg and Baumstark-Khan (2007), the primary components of radiation in interplanetary space are galactic cosmic rays (GCR) and solar cosmic radiation (SCR). GCR originates from outside of our Solar System and consists of 98% baryons and 2% electrons. The baryonic component consists of 87% protons (hydrogen nuclei), 12% alpha particles (helium nuclei) and approximately 1% of heavier nuclei with atomic numbers up to 92 (uranium). These heavier nuclei include highly energetic, heavy, charged particles known as HZE particles. Although ^{56}Fe ions, as a specific type of HZE particle, account for less than 1% of the GCR particle fluxes, they contribute significantly to the total radiation dose received by individual cells exposed to GCR due to the fact that the dose to an individual cell is proportional to the square of the particle's energy dependent effective charge (Katz et al., 1971).

SCR consists of low energy solar wind particles that flow constantly from the Sun and the highly energetic solar particle events (SPEs) that originate from magnetically disturbed regions of the Sun, which sporadically emit bursts of energetic charged particles (Wilson et al., 1999; Smart and Shea, 2003). SCR is com-

posed predominately of protons, with a minor contribution from helium ions (~10%) and an even smaller contribution from heavy ions and electrons (~1%). SPEs are unpredictable, develop rapidly and usually last for no more than several hours, although some SPEs may continue for several days. Since protons are the major component of SPE radiation, ground-based SPE radiation research is focused on the biological consequences of proton radiation at the appropriate energies, doses, and dose-rates expected during an SPE. A large fraction of the protons during an SPE are in the range of around 50 MeV, but there are also varying levels of protons of higher energies characterizing each individual SPE (NCRP, 1989; NCRP, 2006).

Exposure to space radiation may place astronauts at significant risk for acute radiation sickness (ARS), significant skin injury and numerous other biological effects resulting from exposure to radiation from a major SPE, which normally includes some HZE particles, or combined SPE and GCR. Doses absorbed by tissues vary for different SPEs and model systems have been developed to calculate the radiation doses that could have been received by astronauts during previous SPEs (Hu et al., 2009). For instance, it has been estimated that the August 1972 SPE could have delivered

doses of approximately 2.69 Gy and 0.46 Gy to skin and blood forming organs (BFO), respectively, in a spacecraft and 32 Gy and 1.38 Gy to skin and BFO, respectively, during extra-vehicular activity (EVA). Depending on the radiation dose, dose rate and quality, exposure to radiation during space missions may immediately affect the probability for successful mission completion (mission critical) or result in late radiation effects in individual astronauts (Hellweg and Baumstark-Khan, 2007). While avoidance of the radiation risk is the best protective strategy, it is nearly impossible to avoid the radiation risk completely for astronauts. Therefore, countermeasures against adverse biological effects of space radiation are necessary for the success of long term space missions. National Aeronautics and Space Administration (NASA) is primarily concerned with the health risks for astronaut exposures to GCR and SPE radiation. SPEs occur with variable tissue dose-rates and doses, which range from 0 to 0.5 Gy/hour and 0 to 2 Gy, respectively, and with skin doses > 5 Gy (Hu et al., 2009). NASA has determined that the likelihood of acute risks during internal vehicle activity is extremely small; however, there are scenarios during lunar, trans-lunar or Mars EVAs in which ARS may occur.

Acute radiation sickness has a sequence of a phased syndrome that varies with radiation dose, dose rate, quality and individual radiation sensitivity (Hellweg and Baumstark-Khan, 2007), which can include nausea, vomiting, diarrhea and fatigue. These effects are manifested at approximately 4 to 24 hours post-exposure for exposures at sub-lethal doses, with a latency time inversely correlated with dose. Since exposure to proton radiation, which represents the major type of radiation in an SPE, is known to induce abnormalities in leukocytes, erythrocytes and platelets (Gridley et al., 2008), there is also a reasonable concern for compromised immune functions, especially in the microgravity environment in space (Sonnenfeld et al., 1992; Sonnenfeld and Shearer, 2002; Sonnenfeld, 2002, 2005). Space flight is known to alter immune responses, and the causal factors include the stress due to increased radiation exposure (Shearer et al., 2005; Uri and Haven, 2005; Setlow, 2003) and microgravity and non-load bearing status (Aponte et al., 2006; Kita et al., 2004; Sastry et al., 2001; Armstrong et al., 1993). The consistent effects on the immune system observed so far during space travel are as follows: reduction in peripheral T-cell counts and a decrease in Natural Killer (NK) cell number and functionality (Sonnenfeld and Shearer, 2002; Levine and Greenleaf, 1998), decreases in cell-mediated immunity with altered cytokine production (Crucian et al., 2000; Aviles et al., 2003) but normal levels of serum immunoglobulins (Levine and Greenleaf, 1998). An increased susceptibility to infection under space flight conditions has also been observed (Aviles et al., 2003; Mehta et al., 2000). The main concern of an impaired immune system in the closed environment of a spacecraft is the altered ability to control bacterial, fungal, viral, and parasitic invasions (Sonnenfeld and Shearer, 2002; Shearer et al., 2005; Levine and Greenleaf, 1998; Mehta et al., 2000) and the loss of immunosurveillance leading to tumor growth (Lee et al., 2005). Countermeasures that have been considered and/or evaluated for mitigating acute radiation effects on immune system include interferons, which have a profound effect on the immune response both *in vivo* and *in vitro* (Sonnenfeld and Merigan, 1979), an active hexose correlated compound, which activates immune function (Aviles et al., 2008) and enhances resistance to infection (Aviles et al., 2003, 2006), and vitamin and mineral dietary supplementation, as recently reviewed (Kennedy and Wan, 2011).

In addition to acute effects from radiation, there are numerous other major health concerns related to space radiation exposure. In the NASA Human Research Roadmap (A Risk Reduction Strategy for Human Space Exploration), the Integrated Research Plan (IRP) divides the space radiation risks into the following categories: Risk of Acute and Late Central Nervous System Effects from Radi-

ation Exposure, Risk of Acute Radiation Syndromes Due to Solar Particle Events (SPEs), Risk of Degenerative Tissue or other Health Effects from Radiation Exposure, and Risk of Radiation Carcinogenesis. The Degenerative Tissue Risks include adverse radiation biologic effects on the heart, circulatory, endocrine, digestive, lens and other tissue systems (which would include radiation effects on bone, muscle, etc.). It is noteworthy that the International Commission of Radiation Protection (ICRP) has recently issued a report that has important implications for two of the degenerative tissue risks, circulatory diseases and cataracts (Stewart et al., 2012). In this recent review of early and late effects of radiation in normal tissues and organs, it was concluded that for reactions manifesting very late after low total doses, particularly for cataracts and circulatory disease, it appears that the rate of dose delivery does not modify the incidence, and for these two tissues, a threshold dose of 0.5 Gy was proposed (Stewart et al., 2012). For a NASA mission to Mars, fatal cancer risk has been considered the dominant risk in the past (considering the dose from GCR), but circulatory diseases are likely to be of great importance in the newer risk estimates for a mission to Mars (Cucinotta et al., 2013). There have been a number of recent reviews or updates on the status of space radiation research in the research areas of particular concern to NASA for the exploration class missions planned for the future.

For long term space exploration, bone loss and muscle atrophy due to disuse are other major concerns related to the health of the crew (Shapiro and Schneider, 2000; Shapiro, 2006). In ground-based studies, disuse bone loss has been observed in the hindlimb suspension rodent model (Schultheis et al., 2000; Morey-Holton and Globus, 1998, 2002; Morey-Holton et al., 2005). Irradiation with γ -rays exacerbates skeletal microarchitectural changes that are normally found during progressive, postpubertal aging prior to the onset of age-related osteoporosis (Alwood et al., 2012). Radiation exposure may increase the number of osteoclasts and the extent of acute bone loss via increased reactive oxygen species production and oxidative damage, which implies different molecular mechanisms from the bone loss caused by disuse (Kondo et al., 2010). Irradiation with 250 MeV protons followed by hindlimb suspension resulted in an approximately 20% loss of the trabecular bone volume fraction in the tibia and femur of irradiated mice, and the mice receiving the combined treatment with proton radiation and hindlimb suspension generally experienced greater loss of the trabecular bone volume fraction, connectivity density, and trabecular number than either hindlimb suspension or irradiation treatment alone (Lloyd et al., 2012). Irradiation with ^{56}Fe ions, which represents a significant component of GCR (Katz et al., 1971), stimulates osteoclast differentiation even in the absence of osteoblasts, thereby enhancing the sensitivity of bone cells to the effects of radiation (Yumoto et al., 2010). Iron ion radiation contributes to a reduction in compressive strength and partially prevents the recovery of cancellous microarchitecture from adaptive responses of lumbar vertebrae to skeletal unloading in hindlimb suspended mice (Alwood et al., 2010). Thus, irradiation with heavy ions may accelerate or worsen the loss of skeletal integrity triggered by musculoskeletal disuse in the microgravity environment. There are some publications indicating that countermeasures may be helpful to mitigate radiation induced adverse bone effects; for example, α -lipoic acid protects cancellous tissue from the detrimental effects of irradiation (Kondo et al., 2010).

The space radiation risks to the central nerve system (CNS) have been considered to be extremely important in the recent past due to major publications in this field of research. Examples of such publications include studies suggesting that the induction of Alzheimer's disease may be a space radiation risk (Cherry et al., 2012) and attention deficits may arise following exposure to low doses of space radiation (Davis et al., in press). Many other publications have also indicated that there are major CNS space

radiation risks [e.g., Lonart et al., 2012; Suman et al., 2013; Manda et al., 2007, 2008a, 2008b; Rivera et al., 2013; Poulouse et al., 2011; Tseng et al., 2013; Limoli et al., 2007]. Radiation exposure to γ -rays (Hienz et al., 2008) or ^{56}Fe ions (Higuchi et al., 2002; Rabin et al., 2002; Shukitt-Hale et al., 2000, 2007) is also known to have adverse effects on CNS and neurobehavior of irradiated animals, including reduced performance in motor tasks and deficits in spatial learning and memory. Alterations in neuronal function in the HZE particle irradiated animals include reduced responsiveness to agonist stimulation and increased Nigral cell loss, which parallel the neurobehavioral changes associated with aging (Joseph et al., 1998, 2000). The available data suggest that: a) the neurochemical and behavioral deficits after HZE radiation exposure have an apparent threshold below which there are no effects on these endpoints, b) there does not appear to be a dose–response curve for many endpoints, such as upper body strength or radiation induced taste aversion learning, and c) there is no evidence of spontaneous recovery of function that depends upon the integrity of the dopaminergic system after the HZE radiation exposure (Rabin et al., 2004). It has been reported that persistent radiation induced oxidative stress is associated with space radiation induced CNS effects [e.g., Limoli et al., 2007]. In the CNS research area, there have been several publications indicating that countermeasures exist for some of the space radiation induced adverse biological effects, which include melatonin or a metabolite (Manda et al., 2007), lipoic acid (Manda et al., 2008a; Limoli et al., 2007), fruit extracts, which ameliorate deficits in behavior and signaling in rats irradiated with ^{56}Fe ions (Shukitt-Hale et al., 2007), and flavonoid glycosides from Ginkgo biloba, myricetin and quercetin (EGb761) (Barkats et al., 1995), which have been postulated to improve cerebral metabolism, protect the brain against hypoxic damage and scavenge free-radicals (Joseph et al., 1998).

Carcinogenesis has continued to be the major focus of the NASA space radiation risk experimental investigations over the past several years, with most of the investigations not focused on the development of tumors in animals developing from space radiation exposure(s), but instead focusing on various potential surrogate endpoint biomarkers (SEBs) of space radiation carcinogenesis. There are some recent reviews that focus on the development of cancer in animals exposed to space radiation [e.g., Kennedy and Wan, 2011; Bielefeldt-Ohmann et al., 2012; Kennedy, 2009; Shuryak et al., 2011; Weil et al., 2009], some recent individual reports on space radiation induced tumorigenesis [e.g., Weil et al., 2009; Trani et al., 2010; Dicello et al., 2004; Datta et al., 2013], and some new hypotheses/thoughts concerning mechanisms of radiation carcinogenesis [e.g., Hei et al., 2011; Barcellos-Hoff et al., 2013] and risk estimates of radiation induced cancer [e.g., Hall and Brenner, 2012; Hlatky and Hahnfeldt, 2014]. A notable finding in the recent animal carcinogenesis studies is that ^{56}Fe ions were not substantially more effective than γ -rays for the induction of acute myeloid leukemia (Bielefeldt-Ohmann et al., 2012; Weil et al., 2009). It has been pointed out in numerous current and older reviews of space radiation carcinogenesis studies that space radiation induced malignancies are dependent on the species as well as the strain of the species used, and that a major task in this field of research will involve determinations about the appropriate methods to use for extrapolation of the space radiation induced cancer risks from experimental animal studies to humans. One example of the differences observed in space radiation induced cancer studies concerns the development of hepatocellular carcinoma. While exposure to space radiation(s) has indicated a very high incidence of hepatocellular carcinoma in one mouse strain (Bielefeldt-Ohmann et al., 2012; Weil et al., 2009), in other experiments on space radiation induced carcinogenesis using a different strain of mice, a dose of 0.5 Gy from ^{56}Fe ions or 3 Gy from protons had no effects on the develop-

ment of hepatocellular carcinoma (Kennedy et al., 2008). There are some intriguing recent results in the radiation carcinogenesis field of research. Ding et al. (2013) have indicated that there are distinct signatures (transcriptome profiles) in normal human bronchial epithelial cells exposed to γ -rays and different HZE particles. If this effect can be confirmed, it may give rise to studies in which the causative agent can be identified in human malignancies that could have been caused by radiation exposure. While the mechanism(s) involved in space radiation induced carcinogenesis are still unknown, there is evidence that space radiation induced oxidative stress is closely associated with carcinogenesis [e.g., Kennedy and Wan, 2011]. It has been reported that space radiation induces persistent oxidative stress in mouse intestine, which is likely to be associated with intestinal tumorigenesis (Datta et al., 2012a). There is evidence that space radiation induced carcinogenesis can be prevented or mitigated by several cancer chemopreventive agents, which include antioxidants and protease inhibitors [as recently reviewed Kennedy and Wan, 2011], retinoids [e.g. Burns et al., 2001, 2007; Zhang et al., 2006] and fruit extracts (Rabin et al., 2005a, 2005b). In addition, there are a number of new potential cancer preventive agents that have been shown to mitigate *in vitro* SEBs of the space radiation cancer risk; examples include melatonin (Das et al., 2011) and a synthetic triterpenoid, bardoxolone methyl, which protects against space radiation-induced transformation of human colon epithelial cells (Eskiocak et al., 2010).

There is extensive evidence that radiation exposure on earth can give rise to cardiovascular diseases, as recently reviewed (Stewart et al., 2012; Travis et al., 2012, 2014; NCRP, 2011). In the research on heart and circulatory effects resulting from exposure to space radiation(s), it has recently been reported that doses of 2 to 5 Gy ^{56}Fe ion radiation targeted to specific arterial sites in apolipoprotein E-deficient (apoE^{-/-}) mice accelerated the development of atherosclerosis (Yu et al., 2011). In these studies, it was concluded that ^{56}Fe ions can promote the progression of atherosclerotic lesions to an advanced stage characterized by compositional changes indicative of increased thrombogenicity and instability. In numerous other studies, space radiation has been shown to have detrimental effects on many other parameters related to cardiovascular and circulatory diseases, with particularly strong effects leading to endothelial dysfunction [e.g., Soucy et al., 2011] and angiogenesis [e.g., Grabham et al., 2011, 2012, 2013; Grabham and Sharma, 2013].

Risks of other degenerative diseases include radiation induced cataracts, as recently reviewed (Stewart et al., 2012; Blakely et al., 2010; Dynlacht, 2013; Kleiman, 2012; Rehani et al., 2011; Little, 2013; Ainsbury et al., 2009). It is noteworthy that the ICRP has recently proposed lowering the threshold for radiation induced cataracts to 0.5 Gy (Stewart et al., 2012). There have also been some recent studies on space radiation (or other similar types of radiation such as heavy ion-cancer therapy [hadron therapy]) induced cataracts (Dynlacht et al., 2011; Chang et al., 2005, 2007; Blakely and Chang, 2004; Henderson et al., 2010). It has been reported that astronauts have an elevated risk of developing cataracts (Cucinotta et al., 2001; Rastegar et al., 2002), which has been associated with exposure to the high linear energy transfer (LET) GCR present in the space environment.

Current medical treatment for the acute radiation syndrome routinely includes supportive care, antibiotics (quinolones and other agents), cytokine therapy, anti-emetic agents and analgesic agents (Dainiak et al., 2003; Waselenko et al., 2004). Other agents can also be used for the effects of the acute radiation syndrome, such as antihistamines, anti-inflammatories and radioprotectors (Harding, 1988). Several FDA approved anti-emetic drugs, such as Kytril (granisetron), Zofran (Ondansetron), Decadron® (dexamethasone tablets) and Emend (Aprepitant) are known to prevent or alleviate nausea and vomiting in patients or animals exposed

to radiation or chemotherapeutic agents (Hawthorn and Cunningham, 1990; Lofters et al., 1997; Priestman, 1989; Nuth and Kennedy, 2013). A systematic review and meta-analysis of 14 randomized controlled trials, comprising 1451 patients, showed that amifostine (WR2721) significantly reduced the side effects of radiation therapy (Sasse et al., 2006). In the animal studies, treatment with amifostine was shown to protect against DNA damage in cisplatin treated murine peripheral leukocytes (Prieto Gonzalez et al., 2009), reduce changes in nucleolar morphology induced by cisplatin treatment (Muller et al., 1993) and protect against cyclophosphamide-induced disruption of taste (Mukherjee et al., 2013) and methotrexate-induced small intestinal mucositis (Chen et al., 2013), as well as inhibit tumorigenesis (Carnes and Grdina, 1992). Unfortunately, the severe side effects of amifostine have limited its use in the space program for astronauts as well as in other human populations exposed to radiation. PrC-210 is a new aminothiols that has shown no detectable nausea/vomiting or hypotension side effects in the ferret and rat models (Soref et al., 2012), in contrast to the strong side effects of the current aminothiols, amifostine (Rose, 1996; Kligerman et al., 1988); this compound shows promise as a new aminothiols radioprotector.

In the past several years, we have been engaged in research to assess whether there will be adverse acute biological effects similar to those of ARS after exposure to the types of radiation at the energies, doses and dose-rates expected during an SPE. The overall objectives of our studies were to assess the risk of ARS and evaluate countermeasures for ARS, which can develop after exposure to SPE radiation. There is also a reasonable concern for a compromised immune system, due to high skin doses from an SPE, which can lead to burns. Existing evidence suggests that the best animal model for radiation induced vomiting is the ferret (Florczyk et al., 1982; King, 1988) whereas the best animal model for radiation induced skin changes is the pig (Hamm et al., 2000; Zacharias et al., 1997; Hopewell et al., 1993; Archambeau et al., 1971). Mice, on the other hand, are the most frequently utilized mammalian species for evaluation of many radiation induced biologic effects. A major problem concerning the use of mice for studies of SPE radiation is that mice do not vomit in response to irradiation (Yamamoto et al., 2002). Therefore, three species of animals, i.e., ferrets, pigs and mice, were used in our studies to allow interspecies comparisons of the results obtained in the studies in several different areas of research, whereas the effects on vomiting and skin were evaluated only in the most appropriate animal species. Since astronauts will be exposed to space radiation in a microgravity environment, which is known to cause bone loss, muscle atrophy and injury to soft connective tissues in animal models (Shapiro and Schneider, 2000; Schultheis et al., 2000), some of the radiation experiments with mice have been performed with and without partial weight suspension (PWS) at one-sixth G, which is known to be the gravity on the surface of the Moon (Louisy et al., 1994), or hindlimb suspension (HS), a model appropriate for mimicking travel in deep space (Morey-Holton et al., 2005) to evaluate and quantify the possible synergy between radiation and simulated hypogravity on hematopoietic effects associated with ARS.

In the studies in which SPE radiation effects have been evaluated, it was considered extremely important to have comparable dose distributions between the SPE radiation and a standard reference radiation in the animal model systems so that relative biological effectiveness (RBE) values could be calculated (Cengel et al., 2010). SPE radiation is known to result in an inhomogeneous total body distribution, with a considerably higher dose delivered to the skin and underlying tissues than to the internal organs (Hu et al., 2009). These characteristics of an SPE, which result in an unusual dose distribution pattern, make it difficult to compare the results

of SPE radiation with conventional γ -ray radiation. The dose distribution from electron radiation, however, can be manipulated to simulate the dose distribution expected from SPE radiation. Mega-voltage electron beam radiation has been utilized in the pig experiments to accurately reproduce the total dose and dose distribution of SPE protons (Cengel et al., 2010). The dosimetry involved in determining simulated SPE radiation doses in pigs is illustrated in Fig. 1, which shows that the doses to the external organs (e.g., skin, lens) are very high, while the doses to internal organs (e.g., spinal cord, bone marrow) are quite low. The detailed methods for determining the organ doses are described elsewhere (Wilson et al., 2011; Diffenderfer et al., in press). These methods incorporate modern radiation oncology approaches of computed tomography (CT) based Monte Carlo dosimetry into the studies so that acute biological effects in specific organ systems can be determined in animal model systems, and radiation toxicity from various types of SPE radiation exposures can be compared. This approach has also been used to predict the acute biological effects of SPE radiation exposure in astronauts. Ten full body human CT scans in various geometries have been analyzed to determine the impact of physical and environmental factors on organ dosimetry in humans. It has been found that, depending on the organ system of interest (deep vs. superficial) and the fluence/energy profile of the exposure (hard vs. soft event), either the physical size of the astronaut or the fluence/energy profile for the SPE can be the determining factor for radiation dose/toxicity (Cengel, K.A., Schaettler, M.O., and Diffenderfer, E., Unpublished data). In contrast, most of the experiments with mice or ferrets utilizing SPE-like proton radiation involved homogeneous proton radiation exposures with relatively low energies like those present in SPEs; RBE values were then calculated by comparison of the SPE-like proton results with those obtained in similar experiments using γ -ray radiation.

In this review paper, the results of our studies on the biological effects of several different types of space radiation, which include different types of SPE radiation, are discussed. Both acute and chronic effects of space radiation have been evaluated in these studies.

2. Acute radiation effects

The acute radiation effects evaluated in our studies include effects on hematopoietic cells, immune system effects (which include immune system changes resulting from a high dose of SPE radiation to the skin), behavior/fatigue, heart functions, skin effects and organism survival after exposure to a lethal or potentially lethal dose of radiation.

2.1. Changes in hematopoietic cell counts after proton or conventional reference radiation exposures

The changes in peripheral leukocytes in animals post-irradiation have been evaluated in ICR mice irradiated with 225-kVp X-rays (Wambi et al., 2008), γ -rays from ^{60}Co (Maks et al., 2011) or ^{137}Cs (Romero-Weaver et al., 2014; Romero-Weaver et al., 2013a; Sanzari et al., 2011b), protons with energies of 50-MeV (Maks et al., 2011), 51-MeV (Ware et al., 2010), 70-MeV (Maks et al., 2011), 74-MeV (Romero-Weaver et al., 2014; Romero-Weaver et al., 2013a), 78.4 MeV (Sanzari et al., 2013a) and 1-GeV protons (Ware et al., 2010; Wambi et al., 2009) as well as protons with eight different energies between 31 and 75 MeV and simulated SPE protons with energies between 30 and 150 MeV (Sanzari et al., 2014). The changes in peripheral leukocytes have also been examined in ferrets irradiated with ^{60}Co or ^{137}Cs γ -rays and 110-MeV protons (Sanzari et al., 2013d; Krigsfeld et al., 2014) and Yucatan minipigs irradiated with 6-MeV electrons

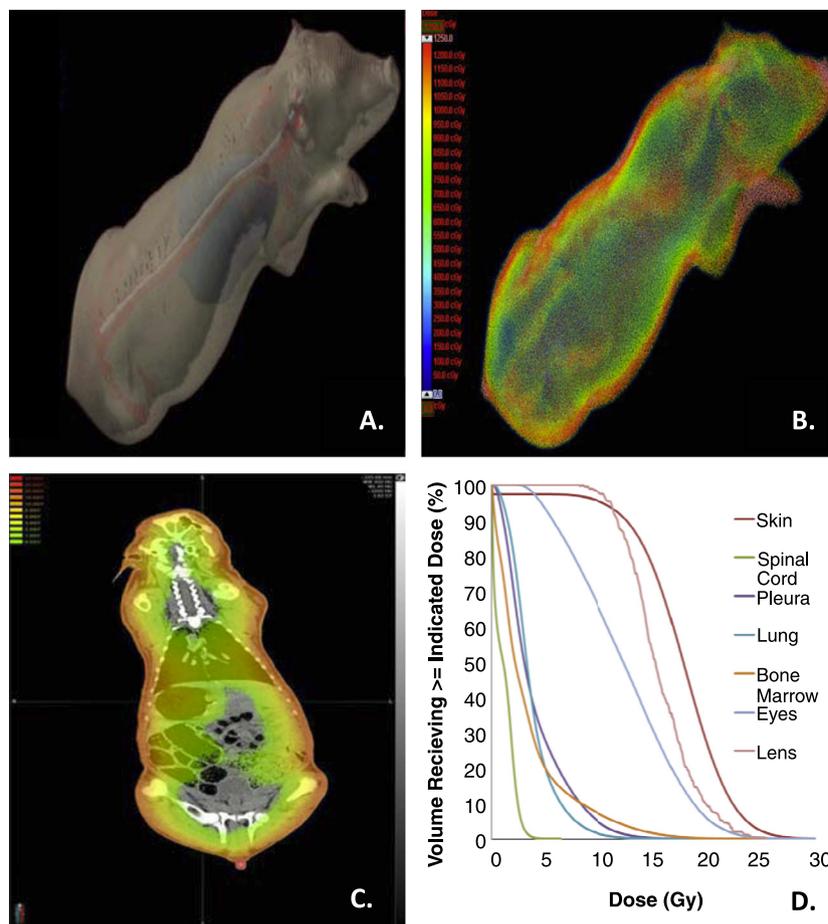


Fig. 1. Simulated dosimetry for Yucatan minipigs. Modern radiation oncology approaches, using CT based Monte Carlo dosimetry, have been incorporated into recent studies to accurately predict specific organ doses from SPE radiation exposure in animals. Pig data are shown in the figures. A: 3D reconstruction from a pig CT image with the skin rendered translucent to allow viewing of the internal organs (i.e., spinal cord [white], bone marrow [red], lung pleura [blue]). The organs were identified on separate CT axial cross sections generating a 3D organ volume from the combined axial contours. (This figure has been reproduced with permission from Radiation Research [Wilson et al., 2011](#).) B: Dose overlay on the 3D rendering of the whole body CT scan. (This figure has been reproduced with permission from Radiation Research [Wilson et al., 2011](#).) C: Dose overlay on a pig CT image (coronal plane cross-section) for 6 + 12 MeV electron irradiation (often called a Heat map or a Dose-Color map) (Doses were simulated for 6 + 12 MeV electron irradiation of a pig using a Monte Carlo based simulation algorithm (Varian Medical Systems, Palo Alto, CA). D: Dose–volume histogram (DVH) figure illustrates organ radiation doses determined using the organ volumes and simulated dose distribution for the pigs receiving a skin dose of 20 Gy (from electrons). Note the high skin dose in the dose–volume histogram (DVH) figure and the fact that the lens dose is also very high. (Images C and D – courtesy of Dr. Eric Diffenderfer.)

([Wilson et al., 2011](#)), 6 + 12-MeV electrons and SPE protons ([Sanzari et al., 2013e, 2013b](#)).

2.1.1. Changes in hematopoietic cell counts in mice after irradiation

In an early study focused on determining the changes in circulating hematopoietic cell counts in mice exposed to SPE-like proton or γ -ray radiation (used as the reference radiation), ICR outbred mice aged 5–6 weeks were exposed to ^{60}Co γ -rays at doses of 0.13, 0.25, 0.5, 1 or 2 Gy or spread out Bragg peak (SOBP) protons (50 or 70 MeV) at doses of 0.25, 0.5, 1 and 2 Gy. The radiation exposures were delivered in a single dose at the low dose rate of 0.5 Gy/hour or the high dose rate of 0.5 Gy/minute. The results demonstrated a dose dependent decrease in white blood cell (WBC) counts in mice exposed to high and low dose rate proton and γ -radiation ([Maks et al., 2011](#)).

In 4–5 weeks old ICR mice, peripheral WBC, lymphocyte and/or polymorphonuclear leukocyte (PMN) counts decreased significantly at 4 and 24 hours after total body irradiation with 1 or 8 Gy of 225 kVp X-rays ([Wambi et al., 2008](#)) or 1 or 5.9, 6.8 or 7.2 Gy of 1 GeV protons ([Wambi et al., 2009](#)). At 24 hours after irradiation with 8 Gy of 225 kVp X-rays, the neutrophil count was decreased to an average of 450 cells/ μl ; neutrophil counts of <500 cells/ μl are clinically significant. At 8 weeks post-irradiation, peripheral WBC and lymphocyte counts in the mice irradiated with 8 Gy of X-rays

were still 64% and 76% below the respective control values ([Wambi et al., 2008](#)), whereas peripheral WBC and lymphocyte counts in the mice irradiated with 5.9 Gy of 1-GeV protons were still approximately 37% and 44% below the respective control values in non-irradiated control animals ([Wambi et al., 2009](#)). In contrast, the PMN/neutrophil count was fully recovered by 8 weeks post-irradiation with 8 Gy of 225 kVp X-rays ([Wambi et al., 2008](#)).

In a separate study performed with male ICR mice aged 4–5 weeks, exposure to 1 GeV proton radiation administered in a single dose at low (5 cGy/minute) or high (50 cGy/minute) dose rates, or in five fractionated doses at the low dose rate resulted in significant and dose dependent decreases in peripheral WBC and lymphocyte counts at 24 hours post-irradiation ([Ware et al., 2010](#)). However, the difference among animals irradiated with the five fractionated doses, or in a single dose at low or high dose rate, did not reach statistical significance at any of the doses evaluated, and neither the WBC counts nor the lymphocyte counts in the animals irradiated with 2 Gy 1-GeV protons in the five fractionated doses, or in a single dose at low or high dose rate, were significantly different from the animals irradiated with 2 Gy of 51.24-MeV protons administered at a low dose rate (5–7 cGy/minute), although they were all significantly below the control WBC and lymphocyte counts in sham irradiated animals. These results suggest that the effect of proton irradiation on the WBC and lymphocyte counts in

the irradiated mice is not altered by dose fractionation, dose rates or proton energy in the ranges evaluated (Ware et al., 2010).

Some experiments were performed to determine whether age or sex/gender differences affected the ability of SPE-like radiation to affect circulating hematopoietic cell counts in mice. To determine the effects of age on this endpoint, 1-year old ICR mice were exposed to proton or γ -ray radiation at doses of 0.0, 0.5, 1.0, or 2.0 Gy, with a dose rate of 0.5 Gy/min (Maks et al., 2011; Romero-Weaver et al., 2013b). Whole blood samples were collected up to 30 days post-irradiation and complete blood counts were analyzed using automated technology as previously described (Maks et al., 2011; Romero-Weaver et al., 2013b; Romero-Weaver and Kennedy, 2012). A comparable experiment was performed in young ICR mice, aged 6–8 weeks. Statistical analyses and RBE values were determined as previously described (Romero-Weaver et al., 2013b) for the experiments performed in both young and aged mice. The older mice in this experiment were specifically aged to simulate healthy, non-smoking, middle-aged astronauts. For example, the preferred age range for European Space Agency applicants is 27–37 years old. The decline in WBCs, neutrophils, lymphocytes and granulocytes were not different using aged mice as compared to the reduction observed in young mice (Sanzari, J.K. and Kennedy, A.R., Unpublished data). The lymphocyte nadir was at 2 days post-proton irradiation, with counts as low as 50%, 60%, and 77% of the control values after exposure to 0.5, 1.0 and 2.0 Gy protons, respectively. The decline in lymphocyte counts after γ -ray radiation exposure was similar to that observed after the proton radiation exposure, which were also comparable to the decline observed in young mice. The results for the granulocyte counts were also consistent between the aged and young mice, with a bimodal decline observed for the first nadir at 4 days post-irradiation (γ -ray or proton) and the second nadir at 16 days post-irradiation. The RBE values at different time points and for each dose were calculated in the aged and young mice. It was observed that the RBE values were not significantly different from 1.0 in either the young or the aged mice. In similar experiments, the radiation effects on WBCs, lymphocytes and granulocytes were shown to be similar between male and female CHH/HeN mice (Billings et al., in press). The only sex/gender difference observed in these experiments was that non-irradiated male mice had 13% higher platelet counts and more enhanced recovery of platelets on day 16 after irradiation as compared to female mice. Thus, it is conceivable that this difference between male and female mice could influence the response of platelets to total body radiation exposure.

In a study conducted with 5 to 7-weeks old female ICR mice, exposure to protons with 8 energy levels ranging from 30.63 to 74.52 MeV or simulated SPE protons with energies ranging from 30 to 150 MeV at high (0.5 Gy/minute) or low (0.5 Gy/hour) dose rate resulted in significant decreases in peripheral WBC and lymphocyte counts as early as 4 hours post-irradiation (Sanzari et al., 2014). At 24 hours post-irradiation with the 30.63–74.52 MeV protons, the dose response curves were nearly identical between the mice irradiated at the low and high dose rates for WBCs or lymphocytes. In a separate experiment performed in the same series of experiments, the mice were irradiated with protons at a very low dose rate of 0.28 cGy/minute (17 cGy/hour), and the results were similar to those of the mice irradiated at the high dose rate (Sanzari et al., 2014). These results again indicate that dose rate in the range evaluated has little or no impact on the suppressive effects of proton radiation on the peripheral WBC or lymphocyte counts in irradiated mice.

During space missions, astronauts are potentially exposed to SPE radiation in a reduced gravity environment. Thus, several experiments have been performed to determine the effects of simulated microgravity on blood cell counts with or without additional exposure to space radiation [e.g., Wilson et al., 2012]. To

evaluate the impact of hypogravity on the effect of SPE radiation on immunological function, experiments were performed with 6–8 weeks old female ICR mice that were irradiated with 0.5, 1 or 2 Gy of γ -rays with or without hypogravity simulated using the PWS model, described by Wagner et al. (2010). The combination treatment with PWS and γ -ray irradiation decreased total splenic lymphocyte viability in a dose dependent manner, and the suppressed splenic lymphocyte viability in groups exposed to a 2 Gy dose of radiation persisted for 4 days, which was the last time point evaluated in the study (Sanzari et al., 2011b). In addition, the viability of splenic lymphocytes was significantly lower in the mice that received a 1 Gy dose of γ -rays in combination with PWS treatment than in the mice that received a 1 Gy dose of γ -rays without PWS treatment on Day-1 or Day 4 post-irradiation. Treatment with PWS alone did not significantly affect the splenic lymphocyte viability at any of the time points evaluated up to 4 days. These results suggest that simulated hypogravity might have made splenic lymphocytes more sensitive to the cell killing effects of radiation. In addition, results from these studies indicated that T cell activation was decreased in the irradiated mice (1 or 2 Gy) with or without simulated hypogravity (PWS). Similar results were observed in experiments using mice exposed to SPE-like proton radiation with and without simulated hypogravity produced by HS (Romero-Weaver et al., 2014; Sanzari et al., 2013a). In these experiments, mice were suspended prior to and after SPE proton radiation exposure and total leukocyte numbers and splenic lymphocyte functions were evaluated on days 4 or 21 after the radiation exposure with and without HS. Splenic lymphocyte subpopulations were altered at both time points investigated. At 21 days post-exposure, T cell activation and proliferation were assessed in isolated lymphocytes. In these studies, T cell activation was suppressed in the proton-irradiated animals and in the irradiated animals exposed to HS. From both types of experiments described above, the results suggest that these irradiated animals with or without additional exposure to simulated microgravity would have immune system suppression resulting from the lack of T cell activation. However, the peripheral blood cell (lymphocyte and granulocyte) counts were significantly higher in proton irradiated mice with HU treatment than without HU treatment and the HU treatment did not significantly interact with the proton radiation dose in the blood cell count data, indicating that the effects of radiation and hypogravity on peripheral leukocytes were simply additive (or subtractive) with no significant synergy (Romero-Weaver et al., 2014).

To determine the RBE values for the effects of SPE protons in hematopoietic cells, several studies were performed in mice using 21-MeV electrons (Gridley et al., 2011), ^{60}Co γ -rays (Maks et al., 2011) or ^{137}Cs γ -rays (Romero-Weaver et al., 2013a, 2013b) as the reference radiations. In male ICR mice irradiated with 2 Gy of 70-MeV protons or 21-MeV electrons, peripheral WBC, lymphocyte and granulocyte counts decreased significantly in the irradiated mice as compared to the sham irradiated controls, but the differences between the proton and electron irradiated mice were not statistically significant for WBCs, lymphocytes or granulocytes (Gridley et al., 2011). In 5 to 6-week old female ICR mice irradiated with ^{60}Co γ -rays, 50-MeV protons or 70-MeV protons at low (0.5 Gy/hour) or high (0.5 Gy/minute) dose rate, the peripheral WBC count decreased in a dose-dependent manner at 24 hours post-irradiation and the RBE values for 50-MeV and 70-MeV protons at either low or high dose rate were not significantly different from 1 with ^{60}Co γ -rays as the reference radiation at the corresponding dose rates (Maks et al., 2011). In a separate study performed with 6-week old female ICR mice, the neutrophil count was monitored for 30 days post-irradiation with 0.5, 1 or 2 Gy of ^{137}Cs γ -rays or 74-MeV protons at dose rates of 0.44 Gy/minute and 0.5 Gy/minute, respectively (Romero-Weaver et al.,

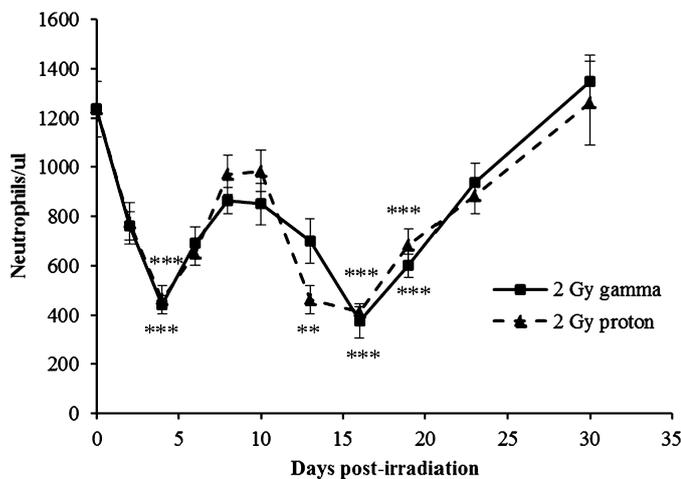


Fig. 2. Changes in neutrophil counts of mice exposed to a 2 Gy dose of SPE proton or γ -ray radiation. As shown in the figure, the effects of SPE like proton radiation on circulating neutrophil counts of mice are approximately the same as those of γ -ray radiation. In this figure, the absolute neutrophil counts are given at various times post-irradiation. In radiation therapy patients, when the white blood cell counts fall below the level of 500 cells/microliter, it would trigger a medical response and the patients would be considered as candidates for countermeasures (e.g., Neulasta treatment). Thus, after irradiation of the mice with either SPE-like proton or γ -ray radiation, the neutrophil counts fall to critically low values (< 500 cells per microliter). (Courtesy of Dr. Ana Romero-Weaver; data from Romero-Weaver et al., 2013b.)

2013a). For the 2 Gy γ -ray and proton dose groups, the neutrophil counts decreased by approximately 65% and 70%, respectively, at the first nadir, which occurred on Day-4 post-irradiation, and then fully recovered by Day-10 post-irradiation to levels that were not significantly different from the pre-irradiation control level. The neutrophil counts for the 2 Gy γ -ray and proton dose groups decreased again by approximately 80% and 75%, respectively, at the second nadir, which occurred by Day-16 post irradiation, and then fully recovered again by Day 23 post-irradiation. Comparable results were observed in similar studies utilizing 74-MeV protons and ^{137}Cs γ -rays in which the changes in WBC counts were evaluated over time (Romero-Weaver et al., 2013b). The results from the studies in mice indicate that proton radiation is not more effective than the commonly used reference radiations with respect to effects on hematopoietic cells (Romero-Weaver et al., 2013a, 2013b).

Overall, the results show that the RBE values are not significantly different from 1 when the effects of SPE-like protons are compared to those from the reference radiations (γ -ray or electron radiation) in mice, and that SPE-like protons and γ -ray radiation result in almost identical dose–response curves over time, as illustrated in Fig. 2 [data in this figure are from Romero-Weaver et al., 2013b].

2.1.2. Changes in peripheral leukocyte counts in ferrets after irradiation

In ferrets irradiated with up to 2 Gy of ^{60}Co γ -rays or 110-MeV protons at a high dose rate (HDR) of 0.5 Gy/minute or a low dose rate (LDR) of 0.5 Gy/hour, the white blood cell (WBC) count decreased significantly within 3 hours after the radiation exposure, and the average magnitude of the WBC decrease in the groups irradiated with 2 Gy of γ -ray or proton radiation at 48 hours post-irradiation was approximately three times the decrease observed at 3 hours post-irradiation (Sanzari et al., 2013d). The lymphocyte count also decreased significantly within 3 hours after irradiation with ^{60}Co γ -rays or 110-MeV protons, but the magnitude of the decrease was similar at the two time points (3 and 48 hours) post-irradiation. In contrast, the neutrophil count increased significantly at 3 hours post-irradiation ($p < 0.001$) and then decreased

significantly at 48 hours post-irradiation ($p < 0.001$), both in a dose-dependent manner.

In addition to the radiation dose, the dose rate also affected the WBC and neutrophil counts at 3 hours, but not at 48 hours post-irradiation, when the WBC and neutrophil counts in the groups irradiated at HDR were lower by approximately 16% and 32%, respectively, as compared to the WBC and neutrophil counts in the groups irradiated at the LDR (Sanzari et al., 2013d). The dose rate effects for the WBC and neutrophil counts were small, as compared to the magnitude of the effect of radiation dose at 3 hours post-irradiation, and disappeared by 48 hours post-irradiation, suggesting that the higher dose rate might have only accelerated the onset of the radiation effects, but did not affect the overall magnitude of the radiation effects that developed at the later time points. Given the fact that the dose rate effect was not observed at 48 hours post-irradiation, when more pronounced losses of circulating WBCs, neutrophils and lymphocytes were observed in the irradiated ferrets, the dose rate effect probably did not have a biologically meaningful impact on the blood cell counts in the irradiated animals.

RBE values were determined for 110 MeV protons using ^{60}Co γ -rays as a reference radiation and peripheral leukocyte counts in the irradiated ferrets as the biological endpoints. The RBE values derived from the WBC counts for 110 MeV protons delivered at the high or low dose rates at 3 and 48 hours post-irradiation ranged from 1.19 to 4.02 at 0.75 Gy and declined with the increase in the radiation dose to a narrow range of 0.59 to 1.04 at 2 Gy (Sanzari et al., 2013d). The RBE values calculated from the lymphocyte count data for the 110 MeV protons at the same time points and dose rates were within a range of 0.83 to 1.41 at 0.75 Gy and showed a slightly downward trend with the increase in the proton radiation dose to a narrow range of 0.67 to 0.84 at 2 Gy. With only a few exceptions, a similar downward trend was also observed for the RBEs based on the neutrophil, monocyte and eosinophil counts in the irradiated ferrets. These results suggest that 110-MeV protons might be more effective than ^{60}Co γ -rays in reducing the peripheral leukocyte counts at the low end of the radiation dose range evaluated in ferrets.

While the early radiation induced changes in the blood cells of ferrets were similar to those in the irradiated mice, the later portion of the time course was quite different between mice and ferrets. In the irradiated mice, the initial sharp decrease in WBC counts post-irradiation is followed by a gradual recovery to baseline levels by 30 days post-irradiation. However, the recovery in the WBC counts did not occur in ferrets exposed to radiation at doses of 1.5–2 Gy; 2 Gy was the highest dose of proton or gamma radiation evaluated in ferrets. This was shown to be due to the development of disseminated intravascular coagulation (Krigsfeld et al., 2014), as discussed below.

2.1.3. Changes in peripheral leukocyte counts in Yucatan minipigs after irradiation

In the Yucatan minipigs irradiated with 6-MeV electrons at a total skin dose of 25 Gy, the WBC count decreased significantly on day-1 post-irradiation, and then recovered by day-7 and increased significantly above the pre-irradiation level by day-30 after irradiation (Wilson et al., 2011). The WBC count did not change significantly in any other dose groups irradiated with electrons at a dose of 15 Gy or below. The lymphocyte count in the minipigs decreased significantly as early as 4 hours post-irradiation with 6-MeV electrons at skin doses of 15 or 25 Gy and then recovered to the pre-irradiation level by day-14 and day-7 for the 15-Gy and 25-Gy dose groups, respectively. The neutrophil count did not change significantly after exposure to the electron irradiation in any dose groups at any time points except for a two-fold increase

in the neutrophil count in the 25-Gy dose group at day-30 after irradiation.

In the Yucatan minipigs exposed to radiation with 6 + 12 MeV electrons, which is a suitable reference radiation with comparable body dose distribution as the SPE radiation (Cengel et al., 2010), the WBC count decreased significantly within a day post-irradiation in the 10, 15 and 20 Gy dose groups, but not in the groups irradiated at a dose of 7.7 Gy or below (Sanzari et al., 2013b). Between day-1 and day-7 post-irradiation, the WBC count reached the lowest level, and then recovered slowly thereafter. By day-30, the WBC counts in the 10, 15 and 20 Gy dose groups all recovered to levels that were not significantly different from the baseline value. The significant decrease in lymphocyte counts occurred earlier, to a greater extent and extended to lower dose (i.e., 5, 7.5 and 7.7 Gy) groups than the decrease in the WBC count after the 6 + 12 MeV electron irradiation. Within a day after irradiation with 6 + 12 MeV electrons at doses up to 20 Gy, the lymphocyte count decreased by up to 77%. By day-30 post-irradiation, the lymphocyte count in the 5, 7.5, 7.7 and 15 Gy dose groups recovered to levels that were not significantly different from the baseline value; however, the lymphocyte counts in the 10 and 20 Gy dose groups were still significantly below the baseline level. The neutrophil count in the Yucatan minipigs irradiated with 6 + 12 MeV electrons did not show a consistent pattern of change among different dose groups post-irradiation. Both increases and decreases in the neutrophil counts were observed at different time points post-irradiation with 6 + 12 MeV electrons at skin doses up to 15 Gy, although the changes did not reach statistical significance due to the relatively large variations in the control group and in some of the irradiated groups.

In the Yucatan minipigs irradiated with SPE-like protons with energies of 155 MeV or below, the WBC count decreased significantly within a day post-irradiation with a single skin dose of up to 10 Gy (Sanzari et al., 2013e). Between day-1 and day-4 post-irradiation, the WBC count reached the lowest level, and then recovered slowly thereafter. By day-30, the WBC count was no more than 38.3% below the baseline level for the animals irradiated with 5, 7.7 or 10 Gy doses of protons. By day-90, the WBC count recovered fully for the 5 Gy radiation dose group while remaining 18.7% and 33.5% below the baseline level for the 7.7 and 10 Gy radiation dose groups. Significant decreases in lymphocyte counts occurred earlier and to a greater extent than the decrease in the WBC count for the minipigs irradiated with 5, 7.7 and 10 Gy of protons. On day-1 after irradiation, the lymphocyte count reached the lowest level, which was 73.0%, 79.7% and 89.5% below the baseline level for the 5, 7.7 and 10 Gy radiation dose groups, respectively. By day-30 post-irradiation, the lymphocyte count in the irradiated animals was not more than 34.5% below the baseline level. By day-90, the lymphocyte count recovered fully for the 5 Gy radiation dose group and was not more than 19.5% below the baseline level for the two higher radiation dose groups.

The neutrophil count change in the Yucatan minipigs displayed quite a different time course as compared to the changes observed in the WBC and lymphocyte counts. The neutrophil count increased by up to 79.8% at 4 hours post-irradiation, and then decreased by up to 42.1% on day-1 after irradiation. The neutrophil count reached the lowest level between day-4 and day-14 post-irradiation, and then recovered slowly thereafter. By day-90 post-irradiation, the neutrophil counts in the 7.7 Gy and 10 Gy dose groups were still 29.3% and 48.0% below the pre-irradiation level. The results for the pig WBC counts over a 30 day experimental period following exposure to proton or electron radiation are shown in Fig. 3. It should be noted that, following exposure to electron simulated SPE radiation, the WBC counts return to normal levels by 30 days post-irradiation, but the WBC counts in pigs exposed

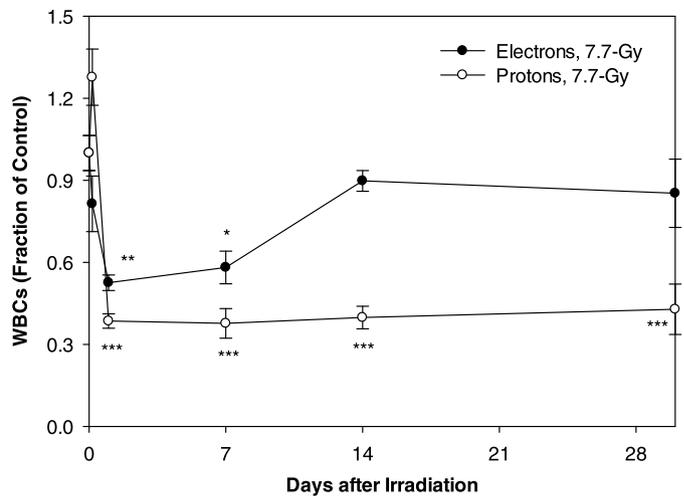


Fig. 3. Changes in WBC counts of pigs exposed to eSPE or pSPE radiation with a skin dose of 7.7 Gy. The pSPE dose involved several different energies of SPE radiation, as described previously (Sanzari et al., 2013e), and the electron exposure involved two different energies of electrons (6 and 12 MeV electrons to result in a dose distribution like that expected for the SPE radiation exposure). The last time point for the evaluation of the changes in the circulating white blood cell numbers was 30 days post-irradiation. It can be observed in the figure that pig white blood cells return to normal levels by 30 days post-exposure to electron radiation, but do not return to normal levels in proton irradiated pigs over the same time period. Significant difference as determined by one-way ANOVA followed by the Tukey Test is indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

to proton simulated SPE radiation do not return to normal levels over this time period.

Based on the WBC, lymphocyte and neutrophil count data for the minipigs exposed to 6 + 12 MeV electron radiation (Sanzari et al., 2013b) and simulated proton SPE radiation (Sanzari et al., 2013e), RBE values were calculated for the effect of simulated proton SPE radiation on leukocytes in irradiated animals. The results show that the RBE value for the simulated SPE radiation varied with both the radiation dose and the time post-irradiation (Sanzari et al., 2013b). For WBC counts, the RBE calculated for the simulated SPE radiation displayed a downward trend with the increase in radiation dose on Day-1, Day-4 and Day-14 post-irradiation. At the 5 Gy proton dose level, the RBE values for the simulated SPE radiation were 2.0, 4.1 and 3.3 on Day-1, Day-4 and Day-14, respectively, after irradiation (Sanzari et al., 2013b). For lymphocyte counts at 4 hours post-irradiation, the RBE for the simulated SPE radiation also showed a downward trend with increasing dose, with the RBE value changing from 9.6 at 5 Gy to 4.6 at 10 Gy. However, the RBE trend for the simulated SPE radiation calculated from the lymphocyte count data was relatively flat on Day-1 and Day-4 and only slightly downward with the increase in the radiation dose on Day-14 post-irradiation. For neutrophils, the RBE for the simulated SPE radiation also displayed a noticeable downward trend with the increase in radiation dose on Day-4 and Day-14. The fitted RBE values were higher than 1.00 at all three simulated SPE radiation dose levels of 5, 7.7 and 10 Gy for WBCs and lymphocytes, and the lower limits of the 95% confidence interval for the RBEs were above 1.00 for all dose levels on Day-1, 4 and 14 except for 10 Gy on Day-1 for WBCs and 7.7 and 10 Gy on Day-4 for neutrophils. In addition, the ED₁₀ and ED₅₀ values for the simulated SPE proton radiation were significantly lower than those for the 6 + 12 MeV electron radiation on Day-1, 4 and/or 14 post-irradiation for WBCs, lymphocytes and/or neutrophils (Sanzari et al., 2013b). These results indicate that simulated SPE proton radiation is significantly more effective than 6 + 12 MeV electrons with respect to the effects on the peripheral WBCs, lymphocytes and neutrophils, especially at the low end of the radiation dose range

evaluated. It was observed that the neutrophils were particularly sensitive to the damaging effects of proton radiation.

2.1.4. Summary of effects of SPE radiation on hematopoietic blood cell counts in ferrets, mice and pigs

For hematopoietic cells, significant decreases in white blood cell counts were observed in mice and ferrets irradiated at high (~0.5 Gy/minute) and low (0.5 to 0.17 Gy/hour) dose rates, starting at doses of 0.5 Gy and up to 2 Gy. In some, but not all, of the studies involving dose-rate comparisons, lowering the dose rate had a small, but statistically significant, sparing effect on the white blood cell count parameters evaluated, but in many other dose-rate experiments performed, such changes were not observed. Thus, over the range of dose-rates evaluated, it is concluded that lowering the radiation dose-rate does not produce sparing effects on hematopoietic cell counts that are of biological significance. At the higher doses of proton or γ -ray radiation, the neutrophil counts in the blood of both mice and ferrets reach critically low levels (< 500 cells per microliter) (Romero-Weaver et al., 2013a, 2013b; Krigsfeld et al., 2014). If such a low value occurred in a patient in a hospital (e.g., following radiation or chemotherapy for cancer), this would trigger a medical response, and suggest the use of countermeasures to increase the level of neutrophils. For the pigs, there were also highly significant reductions in the levels of WBCs following proton or electron SPE radiation exposure, but they did not reach the critically low values observed in the mice or ferrets at any of the high skin doses evaluated.

A major difference in the pig response to the electron and proton SPE radiation was that the neutrophil count did not show a meaningful recovery by 3 months after exposure to 10 Gy of proton SPE radiation (Sanzari et al., 2013e). For pigs exposed to 10 or 20 Gy of electron SPE radiation, the neutrophil count recovered as expected within a month after the radiation exposure (Sanzari et al., 2013b). These results indicate that the pigs might be less capable of repairing the DNA damage caused by the proton radiation exposure than the DNA damage caused by the electron radiation at similar or higher doses. Similar results were observed for mice exposed to proton total body radiation at a dose of 5.5 Gy, which significantly suppressed the neutrophil count at 9 weeks post-irradiation (Wambi et al., 2009). In contrast, the neutrophil count in mice exposed to x-rays at comparable or considerably higher doses (up to 8 Gy) was fully recovered at 8 weeks post-irradiation (Wambi et al., 2008). These results suggest that mouse neutrophils might also be more sensitive to the DNA damaging effects of proton radiation when given at a relatively high dose (5.5 Gy). At a dose of 2 Gy, the neutrophils in the irradiated mice appeared to be equally sensitive to the damage from proton and γ -ray irradiation (Romero-Weaver et al., 2013a, 2013b).

We have calculated RBE values for SPE-like radiations using hematopoietic cell count data from mice, ferrets and Yucatan minipigs irradiated with SPE-like proton radiation and a suitable reference radiation (e.g., γ -rays, x-rays or electrons). A higher RBE value for a given biological endpoint, such as blood cell count, indicates that the SPE-like proton radiation is more effective than the reference radiation in affecting that biological endpoint. In these studies, it was observed that there were: 1) different RBEs for different biological endpoints in the same animal species/strain, and 2) different RBEs for the same endpoint in different species/strains. The RBE values estimated based on the WBC results vary greatly between mice, ferrets and pigs, with the RBE values being greater in ferrets than in mice at times up to 48 hours post-irradiation (Sanzari et al., 2013d), and considerably greater in pigs than in ferrets and mice (Sanzari et al., 2013b). This trend suggests that the RBE values for WBC counts in humans could be considerably greater than those observed in smaller mammals, and SPE proton

radiation may be far more hazardous to humans than previously estimated from studies performed with small animals.

2.2. Immune system effects

2.2.1. Effect of SPE-like radiation on gastrointestinal tract integrity

Numerous studies have been performed to evaluate the effects of SPE radiation on the immune system as a part of research on the acute risks of SPE radiation exposure (Sanzari et al., 2013a, 2013b; Wilson et al., 2012; Li et al., 2014; Zhou et al., 2012). Many of the SPE radiation studies were performed with and without microgravity simulated with PWS or HS (Romero-Weaver et al., 2014; Sanzari et al., 2013a; Wilson et al., 2012; Li et al., 2014; Zhou et al., 2012; Ni et al., 2011). For the studies related to immune system effects, mice were exposed to homogeneous doses of either γ -ray or SPE-like proton radiation. The effects of γ -ray and SPE-like proton radiation were comparable in these studies, and none of the observed effects described below were specific to proton or γ -ray radiation.

The gastrointestinal (GI) tract contains over 10^{12} bacteria, and these bacteria have many important functions including carbohydrate fermentation and absorption, repression of pathogenic microbial growth, and continuous modulation of the gut and systemic immune system. A critical function of the GI tract is the containment of commensal bacteria, which involves the control of bacteria and bacterial product passage across the GI mucosa, known as bacterial translocation; this function can be disturbed in many different diseases. In a study performed with ICR mice at 5–6 weeks of age, irradiation with 2 Gy of 50 or 70-MeV protons resulted in a transient increase of lipopolysaccharide (LPS) in the serum at one day post-irradiation, and the increase was accompanied by increases in acute-phase reactants, such as lipopolysaccharide binding protein (LBP) and soluble CD14 (sCD14), circulatory pro-inflammatory cytokines (including TNF- α , IL-1 β and IL-6), and transient disruption of tight junctions in the GI track, which indicated a transient increase in bacterial translocation across the GI tract and systemic activation of the innate immune system (Ni et al., 2011). HS was also shown to cause a breakdown in the containment of Gram negative bacterial products, as measured by circulating LPS (Ni et al., 2011). The combined treatment of a 2 Gy dose of either γ -ray or SPE-proton radiation with HS led to a greater and more sustained elevation in the level of LPS in the serum. Bacterial translocation is known to increase circulating levels of LPS and other bacterial components, which include bacterial DNA (Jiang et al., 2009). To determine whether the increase in LPS induced a systemic response, LBP, which is a type 1 acute phase protein, was measured in ICR mice exposed to radiation with and without hindlimb suspension. LBP is a circulating protein that binds to LPS of Gram-negative bacteria; it is constitutively present but can be induced to higher levels during various types of infection and inflammatory processes. LBP was increased after treatment with proton radiation or HS and was increased further when these stressors were combined (Zhou et al., 2012). Similar results were observed for sCD14, another very sensitive marker of increased levels of circulating LPS. Circulating levels of interferon-alpha (IFN- α) were measured, and at least additive levels of IFN- α were observed for mice treated with both radiation (2 Gy of γ -ray or proton radiation) and HS. These results demonstrate that circulating LPS, resulting from exposure to SPE-like radiation, HS or both, led to a systemic response. It has been concluded from these studies that there is a synergistic effect when hindlimb-suspended mice are additionally exposed to SPE-like radiation.

To determine the mechanisms involved in the increased bacterial translocation across the GI tract, immunohistochemical staining for the tight junction protein, Claudin-3, was performed on terminal ileum sections of mice, and a significant increase in the

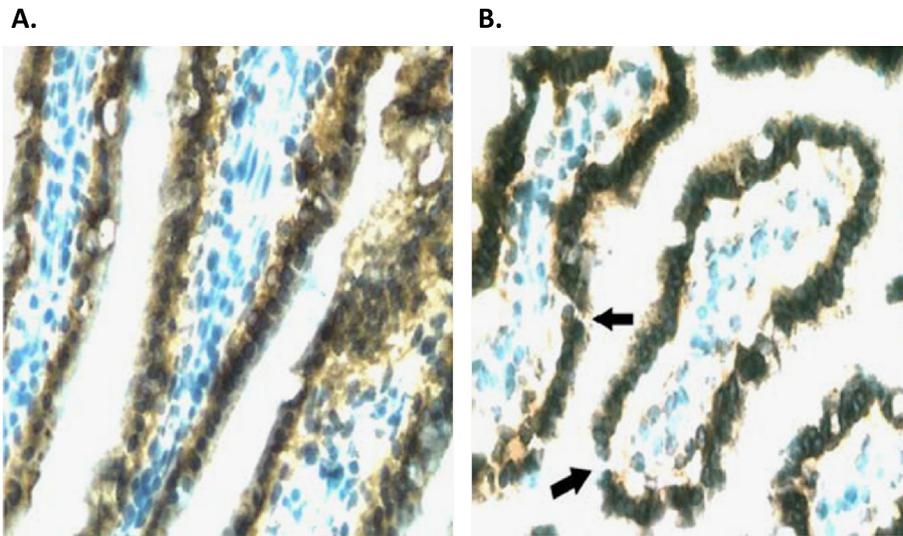


Fig. 4. Proton radiation induces breaks in the GI epithelial barrier. Terminal ileum obtained 2 days post-irradiation from a control mouse (A) or a mouse irradiated with 2 Gy of protons at the low dose rate (B), and stained for Claudin-3. Black arrows show regions of tight junction incongruity. Original magnification 400 \times . Reprinted with permission from Radiation Research (Ni et al., 2011).

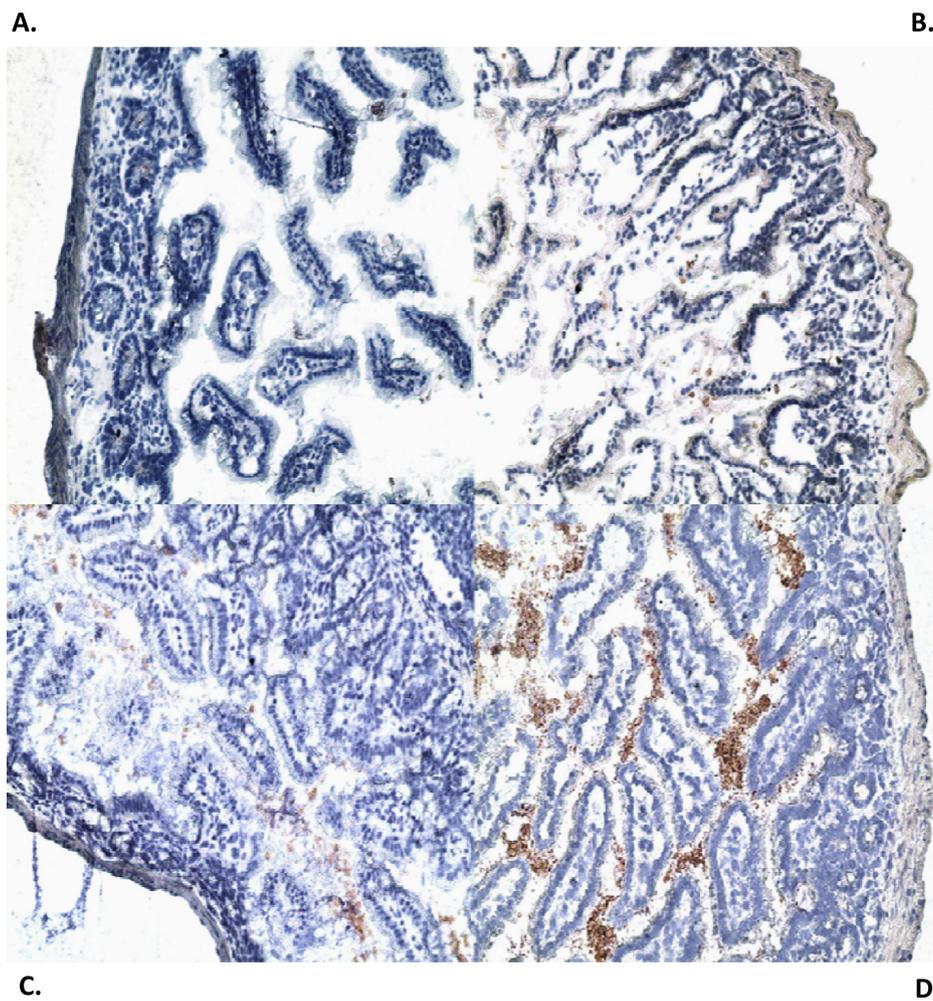


Fig. 5. SPE-like proton radiation and hindlimb suspension lead to the accumulation of LPS in subepithelial regions of the ileum. Terminal ileum obtained 4 days post irradiation and/or 6 days post hindlimb suspension or from control animals was stained for LPS using a mouse mAb specific for *E. coli* LPS. A: represents ileum from a control mouse, B: ileum for a mouse irradiated with 2 Gy of 70 MeV protons, C: ileum from a mouse subjected to hindlimb suspension, and D: ileum from a mouse subjected to HS and irradiated with 2 Gy of 70 MeV protons. It can be observed in the figure that the amount of LPS accumulated in the subepithelial region of the ileum is considerably greater in the mouse exposed to both HS and SPE proton irradiation than in mice exposed to either HS or SPE proton radiation alone. Original magnifications – 200 \times .

number of breaks and reductions in staining were observed in mice exposed to proton or γ -ray radiation and HS, as illustrated in Fig. 4. These studies indicate that SPE-like radiation and hindlimb suspension induced breaks in the GI epithelial barrier, and suggest that the increased frequency of breaks could be responsible mechanistically for the increase in translocation of bacterial products. To further substantiate this association, the terminal ileum was stained with two antibodies that recognize LPS, a mouse monoclonal antibody against *E. Coli* (j5) LPS and a goat anti-lipid A IgG that cross-reacts with *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli* 0157, *Salmonella enteritidis*, *Enterobacter aerogenes*, *E. Hermanii*, *Yersinia enterocolitica* and *Shigella sonnei*. Control animals demonstrated no LPS specific staining in the intervillous space, while low levels were observed in mice treated with SPE-proton radiation or HS alone. For the mice exposed to the combined treatment with SPE-proton radiation and HS, there was a high level of diffuse staining, as shown in Fig. 5. Thus, the conclusion from these studies was that SPE-like radiation and hindlimb suspension induced the accumulation of LPS in subepithelial regions of the ileum.

It has been estimated that astronauts could receive a dose of up to 2 Gy to the bone marrow from SPE radiation (Hu et al., 2009; Townsend, 2005). As discussed above, when a 2 Gy dose of radiation is combined with simulated microgravity, an enhanced and prolonged impairment of commensal bacteria containment was observed. We have identified the mechanism for the loss of containment, which is that radiation plus HS leads to breaks in the tight junctions between GI tract epithelial cells, which results in the migration of LPS into the subepithelial tissue. Potential therapies to treat this immune defect could target the GI defect that leads to bacterial translocation or by reducing the inflammatory activity of translocated bacterial products. The mucosal integrity of the GI tract is maintained by a population of CD4⁺ cells that produce IL-17 (Th17 cells). Their loss is known to be correlated with increased bacterial translocation (Brenchley et al., 2008). Although there are no current therapies that can mitigate the loss of GI Th17 cells, this is an area of research worthy of investigation. Antibiotics can be used to treat the increased bacterial translocation, and they are known to be capable of reducing serum LPS levels (Brenchley et al., 2006).

Numerous immune system alterations have been associated with space flight in humans and in animals during ground-based spaceflight models (e.g. HS), as has been reviewed [e.g., Sonnenfeld and Shearer, 2002; Crucian and Sams, 2009; Gueguinou et al., 2009]. The major effects of spaceflight on the immune system have been well-characterized, and include changes in cytokine production, leukocyte subset distribution and antibody production (Sonnenfeld and Shearer, 2002). Examples of cytokines released in response to stimulation include the following: an increase in anti-inflammatory cytokines and a decrease in TNF- α in LPS stimulated spleen cells (Baqai et al., 2009), reductions in interferon- γ and IL-2 following phorbol 12-myristate 13-acetate and ionomycin stimulation of peripheral blood cells of astronauts (Crucian et al., 2000) and reduced NK cell number and function (Sonnenfeld and Shearer, 2002; Levine and Greenleaf, 1998). Such alterations in immune function are similar to those brought about when there is increased immune activation produced by exposure to pathogen associated molecular patterns (PAMPs), such as LPS (Srinivasan and McSorley, 2007; Bukh et al., 2011; Lee et al., 2009; Jagannathan et al., 2009; Schnare et al., 2001; Medvedev et al., 2006). Specific examples of this are as follows: a reduction in proinflammatory cytokine production by myeloid cells (Medvedev et al., 2006; Kaur et al., 2008), a reduction in antigen-specific T cell effector cytokine responses (Srinivasan and McSorley, 2007) and a reduction in circulating NK cells (Holub et al., 2006). The changes in responses brought about by exposure to PAMPs are known as “tol-

erance”, in which subsequent responses are reduced in quality and quantity, which is thought to protect the host by limiting excessive inflammation and preventing septic shock (Medvedev et al., 2006; Kariko et al., 2004). As has been observed in many different disease states (Jiang et al., 2009; Bukh et al., 2011; Lee et al., 2009; Elson et al., 2005; Obermeier et al., 2005; Caradonna et al., 2002; Schafer et al., 2002), continuous or extended exposure to PAMPs can lead to long-lived immune dysfunction. This condition may exist during space flight, as LBP, a well-known marker of immune activation, is known to be elevated in astronauts' plasma (Gueguinou et al., 2009; Kaur et al., 2008). It is possible that the elevated blood levels of LBP resulting from exposure to SPE radiation and simulated microgravity (HS) may bring about immune dysfunctions of the sort that have been observed during and after extended spaceflight.

2.2.2. Effect of SPE-like radiation on skin immune function

Two models were used to evaluate the effects of SPE radiation on skin immune function. One involved Yucatan minipigs, as their skin histological structure is an accurate model of human skin, and the other involved mice, in which in depth investigations of alterations in immune function could be performed. A major difference between the models used involves the depth of radiation penetration. The pigs were exposed to electrons or protons as an SPE-like inhomogeneous dose of radiation, with a relatively high dose delivered to the epidermis and dermis and lower doses delivered to internal organs (Hu et al., 2009), while the mice received proton or reference radiation (γ -rays) exposures as a homogeneous dose of radiation. The pigs were exposed to skin doses as low as 2.5 Gy to up to 25 Gy, which was the highest skin dose evaluated in these studies. It should be pointed out that skin doses for major historical SPEs were calculated to be as high as 32 Gy (Hu et al., 2009); however, such a high skin dose would represent a worst case scenario that would not be expected to occur frequently. Delayed type hypersensitivity (DTH) responses to phytohemagglutinin (PHA) and LPS were measured prior to radiation and at 7, 14 and 30 days post-irradiation. The responses were symmetric and recorded as the average distance across for induration, erythema and ulceration. Since a similar pattern was observed for all doses of radiation used, with significant increases in the response after radiation, but with no dose dependency, we analyzed all radiation dose groups together for the response to control (PBS), PHA and LPS treatments. A significant enhancement in the DTH response to PHA was observed at all post-irradiation time points evaluated. The responses to LPS were not significantly elevated at day 7, but they become statistically significant at 14 and 30 days post-irradiation (Wilson et al., 2011). The appearance of ulceration after radiation exposure was noted for both PHA and LPS treatments. It is assumed that ulceration occurred as part of the enhanced immune response post-irradiation. If radiation was responsible for the ulceration, ulceration should have increased with increasing doses of radiation, which was not the case. Mice were exposed to a homogeneous dose of radiation up to a 2 Gy dose. Mouse skin challenged with intradermal PHA was measured and a similar increase in DTH reactivity was noted after exposure to 2 Gy of proton or γ -ray radiation [Mao et al., 2011 and (Weissman, D., Unpublished data)].

Skin is known to contain high frequencies of FoxP3 expressing regulatory CD4⁺ T cells (Tregs). In the skin from irradiated pigs and mice, immunohistochemical analysis demonstrated a loss of CD3⁺ and CD25⁺ cells. To determine whether loss of Tregs was responsible for the enhanced DTH responses, RNA was isolated from murine skin prior to and at 2, 7 and 14 days post-irradiation for quantitative PCR measurements of CD3, FoxP3 and GAPDH mRNA. The results demonstrate that there was a statistically significant reduction in FoxP3 mRNA at all 3 time points following exposure to 2 Gy of irradiation. Smaller decreases in CD3⁺ cells were

observed, demonstrating that FoxP3 positive cells were being selectively lost (Zhou, Y., Ni, H., Balint, K., Sanzari, J.K., Dentchev, E., Diffenderfer, E., Wilson, J., Kennedy, A.R., Cengel, K.A. and Weissman, D., Unpublished data). Mouse skin was obtained at various time points post-irradiation in the experiment and single cell suspensions of lymphoid cells were obtained by enzymatic digestion for analysis of CD4, CD25 and FoxP3 cells by flow cytometry. Statistically significant decreases in the percent of CD4⁺ T cells expressing CD25 and FoxP3 were observed at all post-irradiation time points evaluated. The greatest loss was observed at 4 days post-irradiation, with a slow increase in Tregs over the next 28 days. The proliferation of skin CD4⁺ T cells increased with the loss of Tregs, demonstrating a functional effect.

The loss of skin Tregs could be due to the cell killing effects of radiation or alterations in trafficking. We examined splenic lymphoid cells and observed that, with the loss of skin Tregs post-irradiation, there was a statistically significant increase in the percent of Tregs in the spleen. The increase in Tregs led to a drop in the proliferation of activated CD4⁺ and CD8⁺ cells. A recent report has indicated that Tregs traffic through skin and that they are the main types of cells exiting skin during inflammation (Tomura et al., 2010). In these studies, it was observed that half of the skin cells that migrated to draining lymph nodes were Tregs at steady state. Tomura et al. also noted that when an immune reaction was induced in the skin, the frequency of Tregs draining to lymph nodes increased significantly and made up the majority of cells exiting the skin (Tomura et al., 2010). In addition, it was found that the increase in Tregs leaving the skin resulted in more suppression of T cell activation in the draining lymph nodes and spleen where Tregs accumulated.

To identify mechanisms for the radiation induced depletion of skin T cells, inflammatory genes in mouse skin obtained prior to and at various time points after mice were treated with a single radiation dose of 2 Gy were analyzed. It was observed that at 6 hours post-irradiation, multiple acute inflammatory markers, CXCL chemokines, were upregulated. At 24 hours and continuing through day 14, chronic inflammatory markers, CCL chemokines, complement and IL-10, were induced. These data indicated that irradiation at a dose of 2 Gy induces long-lived inflammatory changes in the skin, including alterations in chemokines known to attract Tregs to the site of infection, including CCL17 and CCL22 (Riezu-Boj et al., 2011), which are down-regulated. We hypothesize that the radiation induced inflammation establishes an environment that induces T cells, namely of the regulatory phenotype, to leave the skin and reduces their ability to return to the skin. The lack of Tregs in the skin likely results in a loss of control of the inflammatory response induced by PHA and LPS challenges, resulting in enhanced responses post-irradiation. The clinical significance of this for an astronaut exposed to SPE radiation is unknown. Potentially, as skin abrasions occur during space flight, an enhanced inflammatory response in the setting of reduced immune competence due to Treg migration to lymphoid organs could result in a reduced ability to control an infection.

2.2.3. Effect of SPE radiation and hindlimb suspension on immune function measured by bacterial challenge

Infections of the skin, eyes and respiratory tract are common in astronauts: infections have been reported 13 times in the Apollo and 8 times in Skylab missions, and spacecrafts need to be equipped with numerous antibiotics for treatment of such infections (Czarnik, 1988; Dietlein et al., 1975). A number of bacterial infections have been observed in astronauts during or soon after missions, with organisms that do not typically lead to such infections in healthy people. As one example, *Pseudomonas aeruginosa*, which does not ordinarily infect healthy people, was identified as the pathogen that caused a serious life threatening urinary tract in-

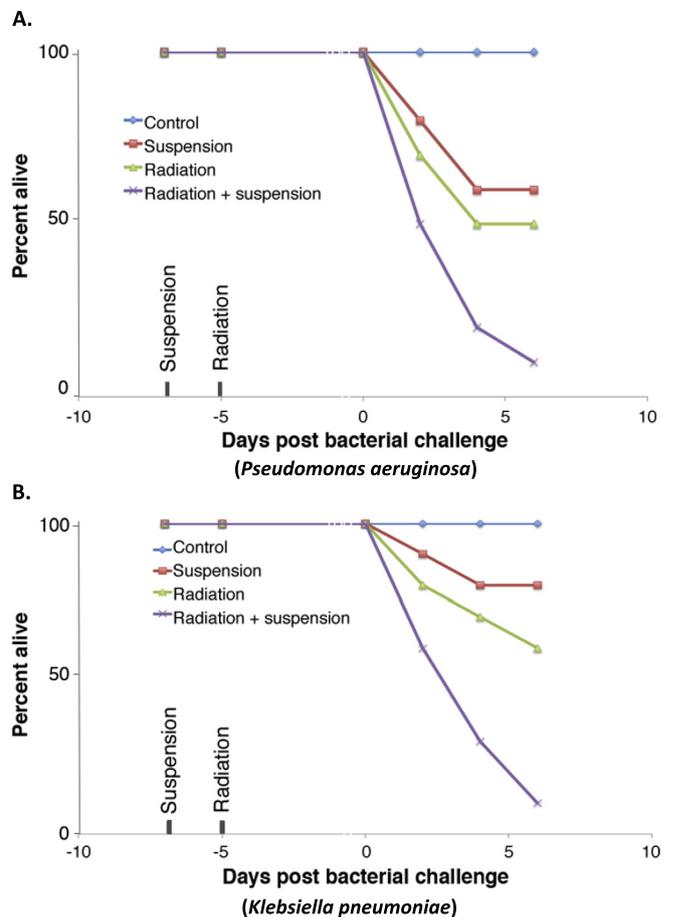


Fig. 6. Survival of mice challenged with bacteria after irradiation with or without hindlimb suspension. C3H/HeN mice were treated with HS, 2 Gy of SPE-like proton radiation, or both, and then the mice were challenged with a non-toxic dose of *Pseudomonas aeruginosa* (Panel A) or *Klebsiella pneumoniae* (Panel B) bacteria. There was a high level of mortality observed in all treatment groups except the control group; these mice were not exposed to HS or radiation, but were given the bacterial challenge. (The images were provided by Drew Weissman, M.D., Ph.D. [data from Li et al., 2014]).

fection in an astronaut during the Apollo 13 mission (Taylor, 1974; Taylor and Dardano, 1983; Taylor et al., 1986, 1997; Taylor and Zaloguev, 1977). As the control of infections during spaceflight is a major problem, much effort has been focused on determining the effects of spaceflight stressors, such as simulated microgravity and SPE-like radiation, on the ability to defend against a bacterial challenge (Li et al., 2014). In these studies, mice were exposed to SPE-like radiation and/or HS, and then challenged with *Pseudomonas aeruginosa* systemically or *Klebsiella pneumoniae* by inhalation. Numbers of bacteria that allow most to all of the untreated animals to survive were used in these studies so that any decrement in immune function could be measured by increased amounts of bacteria in the blood and lung and morbidity. Three different strains of mice were used: ICR mice are an outbred strain initiated in 1948 from Swiss mice, C3H/HeN mice are inbred and have no known defects of polymorphisms that impair DNA repair or the response to ionizing radiation, whereas Balb/c mice have 2 different polymorphisms in DNA-dependent protein kinases (DNA-PKcs) that mediate non-homologous end joining, which results in decreased, but not absent, function (Fabre et al., 2011; Mori et al., 2001; Okayasu et al., 2000). To measure the effect of the HS stress and SPE-like radiation on the ability of different strains of mice to effectively clear a challenge with bacteria, hindlimb suspended and/or irradiated mice were exposed 5 days later to *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*. The mice

were followed daily for signs of systemic and pulmonary infections. The results for all three strains of mice were comparable and indicated that the mice exposed to HS and SPE radiation failed to control a challenge with *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*, which led to a high morbidity/mortality rate (Li et al., 2014), as illustrated in Fig. 6 [data from Li et al., 2014] (note: in these studies, morbidity was the same as mortality). Either SPE radiation or HS alone had some effects on morbidity in these studies, but when combined, they led to almost complete morbidity/mortality. Other studies in this series of experiments led to the following conclusions: 1) similar levels of morbidity were observed after the challenge with *Pseudomonas* bacteria in male and female mice, indicating a lack of gender/sex differences in this effect, 2) a dose of 1.5 Gy of total body radiation impaired the ability of mice to control the bacterial challenge in a fashion similar to that observed for 2 Gy with little difference in the morbidity observed between these two dose groups, but the morbidity differences observed for the 1 Gy dose group compared to those from the control group were not statistically significant, indicating that the threshold dose for morbidity resulting from the bacterial challenge is between 1 and 1.5 Gy, 3) the relative increases in morbidity were similar for all 3 strains of mice, suggesting that the polymorphisms in DNA-PKcs in Balb/c mice did not significantly affect the response to a bacterial challenge, and 4) peripheral blood granulocyte counts were determined in C3H/HeN mice challenged with *Pseudomonas aeruginosa* at time points prior to HS and irradiation and before and after the bacterial challenge. In these studies, there was a reduction in the peripheral blood granulocyte counts observed post-irradiation, which was similar to that described previously (Gridley et al., 2008; Maks et al., 2011; Romero-Weaver and Kennedy, 2012). The numbers of peripheral granulocytes increased as expected after bacterial challenge in control or irradiated mice, but the mice treated with HS, with or without radiation, failed to elevate the blood granulocyte counts as expected (Li et al., 2014). Similar blunting of the peripheral blood granulocyte counts in response to systemic infection in HS animals was also observed in Balb/c and ICR mice (Drew Weissman and colleagues, unpublished data).

2.2.4. Summary of the effects of SPE radiation on the immune system

The effects of SPE radiation on immune system parameters are of great importance to the space program as they are potentially life-threatening at doses that could conceivably be received by astronauts during space travel. It has been observed that exposure to SPE radiation along with HS, with additional exposure to a bacterial challenge, leads to a very high level of morbidity, which is equal to mortality, in the studies performed in mice. The bacterial challenge utilized bacteria known to be associated with astronaut infections and are already part of the spacecraft environment (*Pseudomonas aeruginosa*) or that are part of the normal bacterial flora of the mouth, skin and intestines (*Klebsiella pneumoniae*); the bacterial challenges utilized in the mouse studies described above were performed with bacterial levels that are non-toxic (or minimally toxic) to the normal control mice. Under the conditions described above leading to morbidity/mortality in the mice, death could be prevented by treatment with an antibiotic (enrofloxacin). Enrofloxacin is approved by the FDA as a veterinary antibiotic (marketed by the Bayer Corporation under the trade name Baytril). A similar antibiotic (ciprofloxacin) is in wide use in human populations. While the effectiveness of the antibiotic is outstanding, the major problem associated with the use of antibiotics for bacterial infections in astronauts is that organisms resistant to the treatment can grow out in a very short period of time [as short as a one-week period of time (Personal Communication, Drew Weissman, M.D., Ph.D.)]. Thus, it is expected that in the exploration class missions of the future, the antibiotics are

likely to be less effective with repeated applications over long periods of time. Numerous infections have already been documented in astronauts and are considered a major hazard for spaceflight; some infections have been minor while others have been serious and life-threatening, including a debilitating dental infection and an incapacitating urinary tract infection (Crucian and Sams, 2009; Gueguinou et al., 2009; Taylor and Zaloguev, 1977; Pierson et al., 1994). It is known that spaceflight conditions alter the gene expression patterns, virulence and virulence phenotypes of bacterial pathogens (Nickerson et al., 2004), with evidence of increased virulence under space flight conditions; thus, longer space flights are likely to lead to considerably more serious immunological problems than observed so far in the space program.

The other immunological issue of great importance to the space program is the observation of additive or synergistic-adverse effects caused by SPE radiation exposure and simulated microgravity conditions; interactive effects between SPE radiation and simulated microgravity have been observed for various immunological parameters at doses of radiation that could be received by astronauts during space travel [e.g., Sanzari et al., 2011b, 2013a; Li et al., 2014; Zhou et al., 2012]. The results indicate that, under simulated microgravity conditions, the effects of a given dose of SPE radiation can be considerably more severe than the effects observed for the same dose of radiation in normal, control animals. These results suggest the possibility that, in the space microgravity environment, the effects of a given dose of SPE radiation could be comparable to those observed for a significantly higher dose of radiation.

2.3. Emesis

The early phase of the acute radiation syndrome, which is known as the prodromal syndrome, can include nausea, retching, vomiting, diarrhea, and fatigue (Hellweg and Baumstark-Khan, 2007). These effects often manifest within 1 to 72 hours post-irradiation at sub-lethal doses, with a latency time inversely correlated with dose. Vomiting is the reflexive act of forcefully ejecting the stomach contents through the mouth by coordinated muscle contraction. Published clinical studies have demonstrated that patients receiving total body irradiation or upper-abdominal irradiation often show nausea, retching and vomiting as side effects (Feyer et al., 1998; T.I.G.f.A.R.i. Radiotherapy, 1999). A strong correlation between retching and vomiting events has been established in the ferret model (King, 1990; Andrews et al., 1990). Emetic responses to various pharmacological agents, cytotoxins and radiation have been compared previously among humans and various animal species including nonhuman primates, dogs, cats, and ferrets (King, 1990). Ferrets are considered to be a useful species in emesis research (Florczyk et al., 1982), especially for radiation and cytotoxic drug-induced emesis (Andrews et al., 1990), and data from the ferrets have been used by the Department of Defense to develop a mathematical model for the human emetic response to radiation (McClellan et al., 1992). Another advantage of the ferret model is that the prodromal response appears at lower radiation doses and with an earlier onset time as compared to other species, including humans (King, 1988). Thus, we have chosen ferrets as an experimental model system to determine the effectiveness of protons at the energy, doses and dose rate ranges relevant to the SPE radiation exposures expected during space travel.

In our studies performed with female descended Fitch ferrets aged 12 to 16 weeks, irradiation with ^{60}Co γ -rays or 155-MeV protons at a high dose rate of 0.5 Gy/minute resulted in dose-dependent changes in the endpoints related to retching and vomiting, such as the fraction of animals that retched or vomited, the number of retching and vomiting events, the length of the latency period leading to the first retching or vomiting event and the dura-

tion between the first and last retching or vomiting events (Sanzari et al., 2013c). A dose–response relationship was observed for ferret vomiting and retching at the high dose rate. The minimum radiation doses required to induce statistically significant changes in the retching- and vomiting-related endpoints were 0.75 and 1.0 Gy, respectively; thus, these values are considered the threshold doses for radiation induced retching and vomiting in the ferret model. The RBE of the proton radiation at the high dose rate did not differ significantly from 1. Similar, but smaller and less consistent, changes in the retching- and vomiting-related endpoints were also observed for ferrets irradiated with γ -rays and protons delivered at the low dose rate of 0.5 Gy/hour. Since this low dose rate is similar to a radiation dose rate expected during an SPE, these results suggest that the risk of SPE radiation-induced vomiting is low and may reach statistical significance only when the radiation dose reaches 1 Gy or higher.

Ferrets have also been used previously to study emesis induced by radiation with ^{60}Co γ -rays (King, 1988; Rabin et al., 1992), 0.6-GeV/n ^{56}Fe ions, neutrons (Rabin et al., 1992) and 200-MeV protons (King et al., 1999; Rabin et al., 1994), and the emetic response in ferrets was found to be dependent on the type and dose of radiation. High LET ^{56}Fe particles and fission neutrons were comparable in their ability to produce emetic responses (retching or vomiting) in ferrets with an ED₅₀ of 0.35 Gy and 0.40 Gy, respectively (Rabin et al., 1992), whereas γ -rays were shown to be intermediately effective with an ED₅₀ of 0.77 Gy (King, 1988) to 0.95 Gy (Rabin et al., 1992), and high energy electrons were the least effective, with an ED₅₀ of 1.38 Gy (Rabin et al., 1992). The ED₁₀, ED₅₀ and ED₉₀ values estimated for the fraction of animals that vomited after proton irradiation at the HDR were comparable to the ED₁₀, ED₅₀ and ED₉₀ values after γ -ray irradiation at the high dose rate in our study (Sanzari et al., 2013c) or at a dose-rate of 1 Gy/minute, as reported previously (King, 1988). The ED₁₀ and ED₅₀, but not the ED₉₀, values estimated for the fraction of animals that retched (or vomited) post-irradiation with protons at the HDR were lower than the lower limits of the respective 95% confidence intervals previously reported for γ -rays (King, 1988), suggesting that HDR proton irradiation was more effective than HDR γ -ray irradiation in inducing retching and vomiting.

2.4. Effects of radiation on blood coagulation and the development of disseminated intravascular coagulation

Relatively little information exists in the literature on the effects of radiation on blood coagulation. Blood coagulation involves multiple components, which generate a fibrin-rich blood clot to stop bleeding in a process known as hemostasis. Primary hemostasis starts with the activation of platelets at the wound site by exposing collagen to blood, which allows von Willenbrand factor (vWF) to bind to collagen and tethers platelets to the vasculature wall. The coagulation pathway, which is also referred to as secondary hemostasis, occurs simultaneously on the negatively charged surface of activated platelets to generate a fibrin-rich thrombus (Hoffman and Monroe, 2001). The exposure of tissue factor (TF) at the injury site of blood vessels, and its subsequent binding with Factor VII (extrinsic pathway), initiates a thrombin (Factor II) burst that leads to fibrin clot formation through the activation of a series of vitamin K-dependent serine proteases. It is the activation of coagulation factors, such as Factor V and VIII, by thrombin that drives the development of a stable fibrin clot as a part of the intrinsic pathway (Furie and Furie, 1988; Davie et al., 1991).

Previous studies have demonstrated that radiation can induce vWF secretion from human umbilical vein endothelial cells irradiated in tissue culture (Sporn et al., 1984) and the vWF mRNA levels were increased when either human or bovine endothe-

lial cells were exposed to 20 Gy irradiation with a 6-MeV electron beam at a fixed dose of 2.4 Gy/minute (Jahroudi et al., 1996). It has also been shown that in human peripheral blood mononuclear cells (PBMNCs) after irradiation with a 6-MeV electron beam, TF was up-regulated, which led to a significant increase in PBMNC-associated pro-coagulant activity over a time period of 7 days post-irradiation. Increased cellular TF protein concentration was observed up to 7 days post-irradiation, and microparticle-associated TF activity was increased significantly 3 days post-irradiation as compared with the non-irradiated controls. PBMNC-derived microparticles post-irradiation also initiated the plasma clotting faster than microparticles derived from controls. The radiation induced TF expression and increase in procoagulability of PBMNCs and cell-derived microparticles may represent a possible mechanism by which ionizing radiation enhances blood thrombogenicity (Goldin-Lang et al., 2007). In a study performed with leukoreduced fresh-frozen plasma irradiated with 30 Gy of γ -rays, prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time, antithrombin III, protein C, protein S, vWF, ristocetin cofactor, plasminogen- α 2-antiplasmin, the coagulation factors fibrinogen, FII, FV, FVII, VIII, F IX, FX, FXI, FXII, FXIII, and activated factor XII (FXIIa), D-dimer, fibrin monomer, thrombin–antithrombin complex, prothrombin fragment 1 + 2 (F1+2), plasmin- α 2-antiplasmin complexes, and platelet factor 4 were determined, and PT, aPTT and FVIII activities were found to be decreased significantly, whereas activities of the coagulation factors FII, FV, FVII, FIX, FX, FXII were increased significantly post-irradiation (Weisbach et al., 2007).

To determine whether SPE-like proton radiation could affect blood clotting times, we have performed experiments with 12 to 16 week old descended female Fitch ferrets and demonstrated significant increases in the PT at 3 hours post-irradiation with doses of 1 or 2 Gy (but not 0.25 Gy) of 110-MeV protons delivered at a high dose rate of 0.5 Gy/minute or 0.25 and 1 Gy (but not 2 Gy) of 110-MeV protons delivered at a low dose rate of 0.5 Gy/hour (Krigsfeld et al., 2012). Human PT values are commonly reported as an INR which is defined as the patient's 'test' PT value divided by the laboratory 'normal' PT value, raised to the power of the International Sensitivity Index. INR values were calculated for animals exposed to 2 Gy of 110-MeV protons at both the high and low dose rates and 1 Gy of 110-MeV protons at the low dose rate, which resulted in the greatest PT response to proton radiation in the study. Three out of 10 animals exposed to 1 Gy at the low dose rate had an INR value of ≥ 2.0 and an additional 3/10 of the ferrets had borderline INR values (> 1.75) approaching 2.0 (Krigsfeld et al., 2012), which is considered to be of clinical concern for humans (Yuan et al., 2007; Ng, 2009). The INR values for the animals exposed to 2 Gy protons at the high dose rate were significantly higher than the pre-irradiation levels, although they were still below 2. In addition to the increase in PT, aPPT also increased 3 and 48 hours after 0.25, 1 or 2 Gy of 110-MeV proton irradiation at the low dose rate (Krigsfeld et al., 2012). For the ferrets irradiated with 110-MeV protons at the high dose rate, significant increases in aPPT was not observed in any of the radiation dose groups at 3 hours post-irradiation or in the 0.25 and 1 Gy dose groups at 48 hours post-irradiation. The increase in PT induced by the proton irradiation at the high dose rate was due to Factor VII whereas Factors II, V, VII and IX contributed to the increases in PT induced by proton irradiation at the low dose rate (Krigsfeld et al., 2012). These results demonstrated that proton irradiation significantly increased blood clotting times due to different coagulation factors, indicating potential radiation-induced coagulopathy. The finding that the effects of the proton radiation at the low dose-rate are more severe than those at the high dose-rate on an endpoint is an unexpected finding in radiobiology, as the expectation is that reducing the dose

rate will have a sparing effect, thereby reducing the severity of effect on the biological endpoint being evaluated. Thus, the increased effect of low dose-rate irradiation on ferret blood clotting times is particularly noteworthy.

The blood clotting abnormalities in the ferrets are thought to lead to a condition known as disseminated intravascular coagulation (DIC), which is believed to result in 100% mortality in ferrets irradiated with a 2 Gy dose of either γ -ray or SPE proton radiation (Krigsfeld et al., 2014). The increases in the blood clotting times became more evident in ferrets destined to die from exposure to radiation. The LD₅₀ dose for the development of DIC in ferrets is 1.5 Gy (Krigsfeld et al., 2014).

While irradiation with SPE-like protons was shown to increase prothrombin time and partial thromboplastin time (Krigsfeld et al., 2012), the mechanism for the proton radiation induced hypocoagulation remains to be elucidated. We have hypothesized that the SPE-like proton irradiation activates the coagulation cascade, which would put irradiated subjects in a hypocoagulable state. To test this hypothesis, a separate experiment was performed with 12 to 15 week old descended female ferrets irradiated with 1 Gy of 110-MeV protons at a dose rate of 0.5 Gy/hour, and the results indicate that the radiation exposure resulted in coagulation cascade activation, which was indicated by increases in soluble fibrin concentration in the blood and fibrin clots in blood vessels of livers, lungs and kidneys from irradiated ferrets (Krigsfeld et al., 2013). The soluble fibrin concentration was determined using a rapid soluble fibrin assay that was previously developed and implemented at the Loma Linda University Medical Center to aid in early detection of DIC in emergency room, operating room, or transplant patients (Hay and Bull, 2002). In addition to the activated coagulation cascade, PT and aPTT were also increased after irradiation, which is indicative of the involvement of the extrinsic/intrinsic coagulation pathways. The platelet counts in the irradiated ferrets remained at approximately pre-irradiation values for up to 7 days post-irradiation, indicating that the observed effects on blood clotting times were not platelet-related. The activation of the coagulation cascade is expected to consume clotting factors, which, in turn, leaves the animal deficient in clotting factors. Thus, the increased PT and aPTT values in the irradiated animals might have been due to radiation-induced effects on secondary hemostasis. WBC counts were reduced significantly within 24 hours post-irradiation and they remained reduced up to 7 days post-irradiation with a dose of 1 Gy of SPE-like proton radiation.

DIC is a serious, life-threatening condition in which clotting and bleeding are occurring at the same time, and it is often fatal due to multiple organ failure. Mechanistically, activation of the clotting cascade is expected to decrease the bioavailability of the factors in the blood, thereby increasing the PT/aPTT values, as was observed in the irradiated ferrets (Krigsfeld et al., 2012, 2013). Radiation exposure may significantly decrease leukocyte counts, and the prolonged low WBC, neutrophil, and lymphocyte counts can leave the irradiated subjects at risk for infection, thereby further overwhelming hemostasis and potentially leading to DIC, as has been observed in patients with sepsis. Currently, the mechanism of radiation-induced death at the dose expected to kill 50% of the irradiated subjects (LD₅₀) is thought to be due to bone marrow cytotoxicity (known as the hematopoietic syndrome), which results in a dramatic reduction in the number of circulating hematopoietic cells and the resultant symptoms of infection (from white blood cell loss) and bleeding (presumably from platelet loss) (Dorr and Meineke, 2011). However, studies performed with ferrets have suggested that the death of the animals irradiated at the LD₅₀ dose is due to a consumptive coagulopathy, which is followed by the onset of DIC, since hypocoagulation occurred during early time points post-irradiation when the platelet counts were at normal levels (Krigsfeld et al., 2014). The ferret study results have shown that

exposure to proton or γ -ray radiation produced step-wise changes in hemostasis that begin with the radiation activated clotting cascade, which results in the cleavage of fibrinogen and the formation of fibrin clots. Activation of the coagulation cascade also leads to increased PT and aPTT values due to the consumption of associated factors (Krigsfeld et al., 2012). These changes occurred as early as 3 hours post-irradiation, along with the detectable soluble fibrin in the blood as well as detectable fibrin clots in the blood vessels of irradiated tissues (Krigsfeld et al., 2013). In animals destined to die after irradiation, these abnormal hemostasis parameters became progressively worse. In ferrets exposed to a lower (sub-lethal) radiation dose (e.g., 1 Gy), the abnormal hemostasis characteristics recovered steadily and by day-30 post-irradiation, the clot formation, clot size, and platelet clumping values returned to baseline. These results are particularly important, since the recognition of radiation induced DIC as a cause of death could change the course of actions when the acute radiation syndrome is diagnosed and treated in people exposed to radiation through occupational accidents, radiation terrorism or other catastrophic events (Krigsfeld et al., 2014).

It is noteworthy that experiments performed as part of this project also documented the development of DIC in Yucatan minipigs (Krigsfeld, G.S., Shah, J.B., Sanzari, J.K., Lin, L. and Kennedy, A.R., Unpublished data). Three pigs were irradiated with a 2.5 Gy homogeneous dose of total body x-irradiation. One of these pigs died, and another was euthanized; both of these pigs were diagnosed with DIC. The third pig did not die, but exhibited severe blood clotting abnormalities (greatly increased bleeding times, etc.), as did the two pigs that did not survive. In addition, three pigs were irradiated with a 2 Gy total body dose of SPE-like proton radiation. These pigs exhibited severe blood clotting abnormalities (increased blood clotting times), but they survived the radiation exposure.

2.5. Effects of radiation on fatigue

Several experiments have been performed to evaluate the ability of SPE proton and γ -ray radiation to induce fatigue in mice, as reflected in changes in social exploration, submaximal exercise treadmill and locomotor activity (York et al., 2012a, 2012b; York, 2012). The results of studies on social exploration indicated that low, but not high, dose-rate γ -ray and proton radiation exposures led to comparable transient increases in social withdrawal, and these effects are thought to be due to a combination of restraint stress and radiation. The results for studies on submaximal exercise treadmill indicated that neither γ -rays nor protons impaired performance on this test. In a study performed with 7 to 8-week old male CD-1 mice irradiated with 0.5 or 2 Gy ¹³⁷Cs γ -rays at a dose rate of 44.5 cGy/minute (high dose rate), ⁶⁰Co γ -rays at a dose rate of 0.5 cGy/minute (low dose rate) or protons at dose rates of 0.5 Gy/minute (high dose rate) or 0.5 cGy/minute (low dose rate), locomotor activity was reduced in mice irradiated with γ -rays at the high dose rate but not in the mice irradiated with γ -rays at the low dose rate or with protons, which had a similar macroscopic dose distribution as that from ⁶⁰Co γ -ray exposure, when delivered at either the high or the low dose rate (York et al., 2012a). The γ -ray irradiation also increased hippocampal TNF- α expression, which occurred as early as 4 hours post-irradiation and was followed by subsequent increases in IL-1RA in the cortex and hippocampus and reductions in activity-regulated cytoskeleton-associated protein (Arc) in the cortex. These observations indicate that low-dose ionizing radiation rapidly activates the neuroimmune system, potentially causing early onset fatigue-like symptoms in the irradiated animals.

In these studies related to proton or photon induced fatigue, the results were consistent with a threshold effect, i.e., once a dose sufficient to produce a response is given, additional dose does not increase the magnitude of the response. The magnitude of the

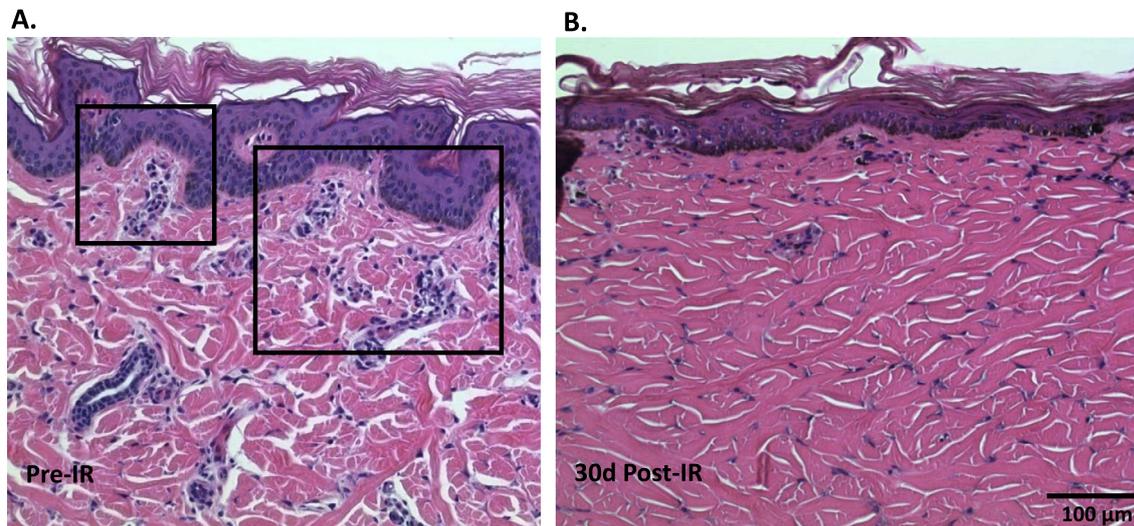


Fig. 7. Loss of blood vessels in the pig dermis after a high skin dose exposure to SPE-like radiation. Pig skin tissue samples were taken pre-irradiation (Panel A) and at 30 days after the pig was exposed to a 10 Gy skin dose of 6 + 12 MeV electron radiation (Panel B). For the skin tissue taken before irradiation, vascular beds are denoted by squares (Panel A). A consequence of exposure to the high skin dose from SPE radiation is that blood vessels disappear in the areas beneath the epidermis (Panel B), such that the blood flow to the cells in this area is reduced. Such areas of reduced blood supply beneath the epidermis occur in interventional radiology patients, and are known to be life-threatening (Balter et al., 2010; NCRP, 2010). Thus, the areas with reduced blood supply must be removed surgically.

response with γ -rays is small, and additional stressors, such as restraint or other manual manipulations, are sufficient to obscure any acute behavioral changes. For those tests in which altered behavior was noted for γ -rays, protons showed a similar trend, which did not reach statistical significance. Therefore, the RBE for proton radiation on this endpoint is assumed to be less than 1, although it could not be defined mathematically due to the lack of a statistically significant trend in the proton irradiated animals.

The data gathered on the ability of SPE radiation to induce fatigue in mice suggest that exposure to low-dose rate ionizing radiation leads to a minimal increase in fatigue, in the form of depressive/anxious behaviors, and that these effects are transient with full recovery within the 24 hour period (York et al., 2012a; York, 2012). These behaviors are likely of equal or lesser magnitude than the depressive/anxious behaviors that are stimulated by the restraint stress necessary to perform the experiments and that is likely to be experienced by astronauts in typical space exploration vehicles. Thus, acutely, SPE radiation up to a 2 Gy total body dose is highly unlikely to increase fatigue or other adverse behaviors over and above baseline levels for astronauts and is therefore highly unlikely to lead to mission critical fatigue.

2.6. Heart functional changes

As part of the experiments designed to determine whether a high skin dose from SPE radiation has adverse effects on the internal organs of pigs, we evaluated changes in the heart brought about by a high skin dose of electron radiation planned to simulate a dose distribution pattern like that expected from SPE radiation. As changes in the left anterior descending (LAD, interventricular) artery can have major effects on heart function, our studies in this area of research focused on LAD function in the pig hearts. The LAD artery provides the blood supply to the mid-region of the heart and is a major site of vessel stenosis. Vessels from control and pigs exposed to electron radiation exhibited a similar relaxation response following treatment with adenosine diphosphate and sodium nitroprusside. There was a reduced relaxation response to bradykinin (BK) treatment in the arteries from hearts exposed to SPE-like electron radiation. In contrast, vessels obtained from control animals exhibited a 20% higher relaxation response with BK, compared with arteries obtained from the irradiated pigs.

Denuded vasculature, isolated from untreated and irradiated animals, was unresponsive to BK treatment, confirming that the BK response is mediated via receptors present on the surface of endothelial cells (Tousoulis et al., 2012). The fact that the relaxation response was lower in irradiated tissue suggests that radiation exposure damages the vascular endothelium. The results of this study demonstrate that radiation has a direct effect on the cardiac vasculature. Intact arteries from hearts taken from irradiated pigs exhibited a reduced contractile force following exposure to BK. BK is an endothelium-dependent dilator that acts directly on endothelial cells, causing them to release nitric oxide, PG12 and perhaps endothelium derived dilating factor(s), which signal relaxation of vascular smooth muscle (Sung et al., 1988). Our results suggest that alterations in vascular function are primarily a consequence of radiation-induced damage to endothelial cells, and that astronauts exposed to a high dose of SPE radiation could be at risk for vascular damage in the heart. While the skin dose was high in this study, the heart dose was only 0.35 Gy. This heart dose is considerably lower than the doses of radiation (0.5–5 G) previously shown to affect endothelial dependent relaxation in rat aorta (Soucy et al., 2007, 2010).

2.7. Skin effects

The skin effects in pigs have been described previously (Wilson et al., 2011) and the immunological changes in pig skin accompanying the skin changes are described above in the section on immunology effects. It has been observed that pigmentation increases with increasing exposure to SPE radiation (Wilson et al., 2011), and a method of quantitation for the melanin changes in irradiated pig skin has been developed (Billings, P.C., Sanzari, J.K., Kennedy, A.R., Cengel, A.K. and Seykora, J.T., Unpublished data). With very high skin SPE radiation doses, there is evidence of blood vessel loss in the pig dermis, as illustrated in Fig. 7. A consequence of this radiation effect is reduced blood flow to these areas of the dermis. It is known that areas of the dermis receiving a reduced blood flow can develop into a “diabetic foot” like state (which needs surgical removal) in patients undergoing interventional radiology procedures (Balter et al., 2010; NCRP, 2010). In humans, the development of such lesions requiring surgical removal takes approximately one year or more and the skin doses associated with

such lesions are high (≥ 10 Gy) (NCRP, 2010). Such lesions could conceivably be a problem during the multi-year exploration class missions planned for the future.

2.8. Increased intracranial pressure and effects potentially related to vision abnormalities evaluated in Yucatan mini-pigs

Vision changes, characterized as a degradation in distant and near visual acuity, have been documented in numerous astronauts who have been involved in long-duration (of six months or longer) space flight (Mader et al., 2011). Although the etiology of the vision alterations is unknown, Mader et al. hypothesized that the optic nerve and ocular changes observed in astronauts could have been caused by prolonged microgravity exposure (Mader et al., 2011). Since radiation exposure in clinical radiotherapy patients is known to be associated with increased intracranial pressure, increased radiation exposures during space travel could also contribute to the alterations thought to be involved in producing the vision abnormalities. We have evaluated the ability of SPE-like radiation in pigs to produce changes like those associated with the astronaut vision alterations, by performing ocular ultrasound examinations, lumbar puncture opening pressure studies and histopathologic examinations of eye tissue taken from pigs irradiated with SPE-like radiation (in the forms of simulated electron (6 + 12 MeV) SPE (eSPE) or simulated proton SPE (pSPE) exposures (Sanzari et al., 2013b)). The dosimetry studies have indicated that the eyes and lenses receive substantial doses of radiation during an SPE; the dose to the lens is roughly comparable to the dose received by the skin of pigs (e.g., see Fig. 1).

2.8.1. Histopathology changes in the eyes of pigs exposed to simulated proton and electron SPE radiation which could be related to vision problems

The retina is a layered structure of neurons interconnected by synapses sending information to the brain via the optic nerve. The retina includes millions of photoreceptor cells, known as rods or cones, which are sensitive to light. Change or damage to the retina can cause loss of vision. Signs of damage to the retina are sudden flashes of light, floating spots, decreased vision, or distorted vision. The ocular histopathology of pigs exposed to pSPE (5–10 Gy) or eSPE (5–20 Gy) radiation was examined. In this study, (Sanzari, J.K., Zeiss C.J., and Kennedy, A.R., Unpublished data) sagittal sections of the entire eye and cross sections of the optic nerve were prepared and qualitative differences (as well as some quantitative measurements) between the eyes of the irradiated and non-irradiated animals were investigated.

It was determined that eSPE radiation exposure resulted in a decreased total retinal width in 20% of the irradiated animals compared to the non-irradiated control animals; the differences observed in these studies between the irradiated and control pigs were statistically significant. Changes in retinal width can cause turbulence in blood flow and may indicate atrophy affecting the nerve fiber layer, ganglion cell layer, outer plexiform layer, and inner nuclear layer of the retina. It was confirmed that the width of the outer plexiform layer was also reduced in some of the animals exposed to eSPE radiation. A reduction in the width of the outer nuclear layer is an indicator of photoreceptor cell loss, which was confirmed by the extrusion of photoreceptor nuclei in the retina. The extrusion of photoreceptor nuclei (an indication of active cell death) into the inner segments was observed in both the eSPE and pSPE animals. The dose–response relationship was evaluated for the extrusion of photoreceptor nuclei induced by pSPE radiation exposure and results indicated a statistically significant slope, establishing that the loss of photoreceptor nuclei in pSPE irradiated pigs was dose-dependent.

Upon evaluation of the optic nerve area, the meningeal sheath area was dilated in 31% of the irradiated animals, and the differences between the measured area of dilation in the irradiated pigs compared to the non-irradiated pigs were statistically significant. Further, an accumulation of lymphocytes, plasma cells, and macrophages around the vessels near the optic nerve was observed, indicating an inflammatory response to radiation exposure; however, the presence of inflammatory infiltrates was not consistent in the irradiated animals (inflammatory infiltrates were present in 10% of the irradiated pigs).

The results described above suggest that SPE radiation may result in radiation-induced retinal atrophy or degeneration; however, long term studies will be necessary to determine whether the loss of photoreceptor cells and changes in retinal width or optic nerve area persist at later time periods.

2.8.2. Ocular ultrasound results of the eyes of the pigs exposed to SPE radiation

Noninvasive measurements such as those obtained from bedside ocular ultrasound examinations have been advocated and utilized in the clinical setting and during spaceflight to give information about changes potentially related to vision abnormalities. Live ocular ultrasound imaging was performed in the pSPE animals approximately 2 months post-irradiation as part of a collaborative study between the investigators at the University of Pennsylvania (Penn) and Wyle/NASA investigators skilled in ocular ultrasound examination techniques. The sonographer at Penn was remotely-guided by an expert in eye/optic nerve imaging, and the remotely guided sessions were conducted in a way that was similar to the “telemedicine” and “telescience” arrangement routinely used during ultrasound imaging sessions on the International Space Station (ISS). The optic nerve sheath diameter was measured using electronic calipers at three distances from the vitreoretinal interface. Animals exposed to 5–10 Gy pSPE radiation exhibited a dose-dependent trend in increased diameter measurements compared to the non-irradiated animals. The measurements recorded for the animals exposed to 5 Gy pSPE radiation resulted in a significant increase ($p = 0.02$ for right eye; $p = 0.05$ for left eye), compared to the non-irradiated group of animals. The 10 Gy dose pSPE radiation resulted in significantly increased sheath diameters as well ($p = 0.002$ for right eye; $p = 0.03$ for left eye) (Sanzari, J.K., Sargysan, A.E., Ebert, D., Garcia, K.M., Shultz, S.M., Seghal, C.M., and Kennedy, A.R., Unpublished data). Ocular ultrasound examinations were not performed on the corresponding animals exposed to eSPE radiation (for which histological changes are reported above).

When considered together, the histopathology and the ocular ultrasound exam results indicate that SPE radiation exposure directly affects the eye structure of animals exposed to 5–20 Gy electron simulated SPE radiation or 5–10 Gy proton simulated SPE radiation. The observed changes in the optic nerve area may be associated with the optic disc edema observed during/post spaceflight reported by Mader et al. (2011).

2.8.3. Opening pressure in pigs exposed to proton and electron SPE radiation

The opening pressure of pigs exposed to either pSPE or eSPE was measured by lumbar puncture procedures and was found to be increased in some of the irradiated animals, with relatively larger increases in animals exposed to larger electron or proton SPE radiation doses (Sanzari et al., 2014). Pigs exposed to lower skin doses of 2.5 to 7.5 Gy eSPE radiation (like the SPE radiation occurring in October 1972) exhibited increased opening pressure values, which lasted up to 90 days post-irradiation (i.e., at the time when the experiment was terminated), suggesting that SPE-like electron radiation resulted in increased intracranial pressure after a radia-

tion exposure with a skin dose as low as 2.5 Gy (Sanzari et al., 2014).

The results in this area of research indicate that exposure to even relatively low skin doses of SPE radiation can result in some of the alterations thought to play a role in astronaut vision alterations (e.g., increased intracranial pressure and increases in nerve sheath diameter). Thus far, astronauts have not been exposed to significant doses of SPE radiation during spaceflight, but it is expected that there will be an increased risk of astronaut exposure to higher doses of SPE radiation in the exploration class missions of the future. It is hypothesized that exposure to SPE radiation along with extended space travel could exacerbate the development of visual changes in astronauts. However, larger numbers of exposed pigs than those used in the studies described above will be necessary to confirm and verify this hypothesis.

2.9. Short-term survival in irradiated animals

In experiments performed with male ICR mice aged 4 to 5 weeks exposed to 5.9, 6.8 or 7.2 Gy of total body irradiation with 1-GeV protons at dose rates ranging from 0.2 to 0.7 Gy/minute, the 30-day survival was 60%, 13.3% and 0%, respectively, and the calculated lethal dose to kill 50% of the irradiated animals was 6.23 Gy (Wambi et al., 2009). In a separate experiment performed with male ICR mice aged 4 to 5 weeks irradiated with 6 or 8 Gy of 225 kVp X-rays, the 30-day survival was 100% and 6.7%, respectively (Wambi et al., 2008); these survival levels are higher than those observed in the 30-day survival studies in the animals exposed to 5.9–7.2 Gy of 1-GeV/n protons. These results indicate that 1-GeV proton radiation is more lethal to mice than X-rays. Similar studies were performed by other investigators in C57BL/6J mice to determine the relative toxicity of HZE radiation (1-GeV/n ^{56}Fe ions) compared to γ -rays or 1-GeV protons (Datta et al., 2012b). In these studies, the LD_{50/30} values for ^{56}Fe ions, protons and γ -rays were reported to be 5.8, 6.8 and 7.25, respectively; the RBE value for the ^{56}Fe ions was 1.25 and for the protons, the RBE was 1.06. It was concluded from these studies that ^{56}Fe ions caused accelerated and more severe hematopoietic toxicity. Of interest in this study was the finding that intestinal crypt cells did not show increased HZE toxicity. In another reported study performed in C57BL/6J mice, it was observed that the LD_{50/30} values for ^{28}Si and ^{12}C ions were 5.17 and 7.34 Gy, respectively (Suman et al., 2012). In these studies, the RBE values for ^{28}Si and ^{12}C ions (compared to the γ -ray data in which the LD_{50/30} was 7.25) were 1.4 and 0.99, respectively.

In studies performed with ferrets, the observed 30-day survival was 100% for ferrets irradiated with up to a 1 Gy dose of SPE-like protons and zero for ferrets exposed to 2 Gy of SPE-like protons (Krigsfeld et al., 2014). The LD_{50/30} for SPE-like proton radiation in ferrets was estimated to be approximately 1.5 Gy (Krigsfeld et al., 2014). The survival curves for γ -ray irradiated ferrets were comparable to those for the proton-irradiated animals, and both the proton and γ -ray irradiated ferrets displayed signs of distress including ecchymosis, petechiae, and hemorrhaging. It was hypothesized that the ferrets were dying of DIC in these studies (Krigsfeld and Kennedy, 2013), which was confirmed by additional evidence collected (Krigsfeld et al., 2014).

3. Long-term effects

3.1. Long-term survival in irradiated animals

In a long-term study in which male CBA/JCR HSD mice aged 8 to 9 weeks were irradiated with 3 Gy of 1-GeV protons or 0.5 Gy of 1-GeV/n ^{56}Fe ions and monitored for 2 years after irradiation, the survival of the mice was significantly lower in mice exposed

to 3 Gy protons ($p < 0.001$) or 0.5 Gy ^{56}Fe ions ($p < 0.05$) than in mice receiving only the sham radiation (Kennedy et al., 2008). The decreased survival in the irradiated mice was accompanied by a significant increase in the rate of development of malignant lymphoma and Harderian gland tumors as well as the fractions of animals with malignant lymphoma or rare tumors (Kennedy et al., 2008, 2011).

3.2. Cataract development

In the long-term experiment with CBA/JCR HSD mice exposed to 1-GeV protons at a dose of 3 Gy or 1-GeV/n ^{56}Fe ion radiation at a dose of 0.5 Gy, the mice were observed daily over approximately two years after the radiation exposure. The animals were then euthanized and lenses were harvested and characterized using an established classification system that assigns discrete scores based on the severity of the lens opacifications. The results showed that exposure to 1-GeV/n proton (3 Gy) or ^{56}Fe ion (0.5 Gy) radiation significantly increased the cataract prevalence and severity in CBA/JCR HSD mice to levels above the baseline levels of age-induced cataract formation in this mouse strain (Davis et al., 2010).

3.3. Cancer development

Malignancy is considered to be a particular risk associated with exposure to the types of ionizing radiation encountered during space flight. In these studies, the ability of protons and highly energetic, heavy particles (HZE particles) to induce carcinogenesis was determined in CBA mice. The major finding of the studies was that there was an increased risk of developing malignant lymphoma and rare tumor types, including Harderian gland tumors, in animals exposed to space radiations (Kennedy et al., 2008, 2011). A significant increase in pre-malignant and malignant lesions of myeloid origin was also observed in mice exposed to 3 Gy proton radiation and 0.5 Gy ^{56}Fe ion radiation. These results indicate that exposure to space radiations can increase the incidence of malignant tumors as well as pre-malignant lesions in mice. These studies have been reviewed recently (Kennedy and Wan, 2011).

4. Radiation induced changes in gene expression

In a study performed with 5–6 weeks old ICR mice, irradiation with 1-GeV protons was shown to increase the mRNA levels for Bax, caspase-9, caspase-8, NF κ B1 and TGF β 1 and protein levels for Bcl2 and Bcl-xL (Sanzari et al., 2011a). The proton irradiation was also shown to induce cleavage of pro-apoptosis proteins, such as caspase-3 and PARP-1, in bone marrow lysates of the irradiated animals. These results confirm the findings that the high-energy proton radiation can induce the gene expression of classical markers of apoptosis, as well as the downstream effectors, caspase-3 and PARP-1. In a separate study performed with ICR mice irradiated with 1.0 and 6.4 Gy of 1-GeV protons or 1.1 and 7.0 Gy of γ -rays to compare the acute effects of radiation on gene expression in radiation-sensitive tissues (e.g., spleen, thymus, bone marrow, testis and the GI tract), the apoptotic responses were found to vary greatly between γ -ray and proton irradiated animals in a tissue- and dose-dependent manner and cell death in the splenic white pulp was consistently lower in the proton-irradiated animals compared to the γ -ray irradiated animals (Finnberg et al., 2008). Both proton and γ -ray irradiation triggered nuclear accumulation of p53, with no significant differences in the majority of the known pro-apoptotic p53-target genes in the spleens of irradiated mice. However, γ -ray irradiation uniquely triggered a pro-apoptotic expression profile in the spleen and Peyer's patches, which exhibited a higher level of apoptosis after γ -ray irradiation than after the proton irradiation despite the increased presence of DNA strand

breaks and phosphorylated-ATM in the spleens of proton irradiated animals. Differences in the acute pro-apoptotic response to proton and γ -ray irradiation correlated with increased expression of the p53-dependent pro-apoptotic gene, Bcl-G, and granzyme B, suggesting that the fate of the cells after proton and γ -ray irradiation may be context-dependent and the triggering of apoptosis in lymphoid cells after irradiation may not be dependent solely on the extent of DNA damage brought about by the radiation exposure.

In female ICR mice irradiated with ^{60}Co γ -rays at low (0.5 Gy/hour) or high (0.5 Gy/minute) dose rates, changes in the expression of genes implicated in oxidative stress, extracellular matrix (ECM) remodeling and selected protein expression profiles in mouse skin were examined using skin tissues harvested at 4 hours post-irradiation (Mao et al., 2011). After irradiation at doses as low as 0.25 Gy, the expression of many genes responsible for regulating the production of reactive oxygen species were significantly altered by more than 2-fold as compared to unirradiated controls. The expression profiles of 18 to 20 of the 84 ECM genes were also significantly altered after irradiation at the low dose rate. As compared to the low dose rate irradiation, the high dose rate irradiation resulted in different ECM gene expression profiles, with the most striking differences observed for genes encoding matrix metalloproteinases. These results indicate that the expression of many genes involved in oxidative stress responses and ECM remodeling may be differentially regulated by high and low dose rate irradiation.

5. Countermeasures and mitigation of space radiation damage

It has been well established that the biological effectiveness of ionizing radiation depends on the LET, which describes the rate of energy loss along the trajectory of ionizing particles (Hall, 2000) and the ion species (Tsuruoka et al., 2005; Tsuruoka et al., 2008). Ionizing radiation damages cells through a combination of direct action, which refers to the direct hit of biologically important targets by the particle radiations, and indirect actions via water-derived free radicals produced by the radiation (Hirayama et al., 2009). While the indirect action plays an important role in the biological effects of low-LET radiations, such as X-rays and γ -rays, its contribution diminishes with increase in LET and the direct action contributes more to the biological effects of high-LET radiation than the indirect action (Hirayama et al., 2009).

From our studies, there have been many publications related to countermeasures for acute radiation effects (Kennedy and Wan, 2011; Kennedy, 2009; Kenedy et al., 2008, 2011; Wilson et al., 2011; Wambi et al., 2008, 2009; Romero-Weaver et al., 2013a, 2014; Krigsfeld et al., 2013; Davis et al., 2010; Sanzari et al., 2011a; Finnberg et al., 2013; Whaley et al., 2013). These publications are briefly described below.

5.1. Radiation induced oxidative stress and antioxidants as countermeasures

Using a dichlorofluorescein (DCF) fluorometric assay that we had previously adapted and standardized to measure radiation induced oxidation in live cells (Wan et al., 2003; Wan et al., 2005a), we have demonstrated that low LET photon radiation, such as X-rays and γ -rays, and high LET radiation, such as 0.6-GeV/n silicon ions and 1- or 5-GeV/n ^{56}Fe ions, as well as 250 MeV protons, are all capable of inducing oxidative stress (Wan et al., 2005b), suggesting that the indirect actions via free radical generation may contribute substantially to the biological effects of both low and high LET radiation. Since the removal of radiation generated free radicals will make them unavailable to damage DNA, proteins and

cell membrane components, antioxidants could be effective countermeasures against radiation induced oxidative stress and other adverse biological effects occurring downstream to the free radical generation.

The agents evaluated in our studies as potential countermeasures for radiation induced oxidative stress included N-acetyl cysteine (NAC), ascorbic acid (or vitamin C), coenzyme Q10, folic acid, glutathione, α -lipoic acid, niacin, L-selenomethionine (SeM), thiamin and vitamin E succinate. NAC is a small molecular weight thiol and a precursor to intracellular cysteine and glutathione (van Zandwijk, 1995), which is a tripeptide small molecular weight thiol shown to be a versatile protector against radiation induced oxidative damage (Bump and Brown, 1990). NAC is also effective in activating NF- κ B and manganese superoxide dismutase (Mn-SOD) gene expression (Das et al., 1995; Murley et al., 2001, 2004); MnSOD is a main mitochondrial antioxidant enzyme with radioprotective properties (Oberley et al., 1987; Clair et al., 1992; Guo et al., 2003). Vitamin C is a water-soluble antioxidant that reacts with highly damaging hydroxyl radicals to form less toxic ascorbate free radicals, which can be detoxified by enzymes that reduce ascorbate free radicals back to ascorbic acid (Rose, 1990). Vitamin E is a lipophilic agent that protects cell membranes from oxidative damage by radiation or other physical or chemical agents (Wolf et al., 1998). Dietary supplementation of vitamins C and E is thought to be important for protection against human diseases associated with free radical damage to cellular DNA, lipids and proteins (Jacob and Burri, 1996). Lipoic acid is a B vitamin that is both lipid and water-soluble and is considered to be a “universal antioxidant” because it can react with hydroxy radicals, singlet oxygen, and peroxy and hypochlorous radicals (Packer et al., 1995). It is an excellent radical scavenger both in the oxidized and reduced form and is known to regenerate other antioxidants from their inactive forms. As examples, lipoic acid plays an essential role in mitochondrial dehydrogenase reactions (Reed, 1974). It also protects cell membranes by reacting with and regenerating vitamin C and glutathione, which in turn recycle vitamin E (Packer et al., 1995). Treatment with lipoic acid has been shown to reduce radiation-induced oxidative stress (Packer et al., 1995; Biewenga et al., 1997; Marangon et al., 1999) and hematopoietic tissue damage in irradiated mice (Ramakrishnan et al., 1992). Treatment with lipoic acid in combination with vitamins C and E has been shown to protect against lens damage caused by low dose irradiation (Bantsev et al., 1997). Selenium is an essential trace element for maintaining activities of the important antioxidant enzymes, thioredoxin reductase and glutathione peroxidase (Michiels et al., 1994; Mustachich and Powis, 2000). Together with vitamin E, it protects cell and organelle membranes from oxidative damage, facilitates the union between oxygen and hydrogen at the end of the metabolic chain and the transfer of ions across cell membranes (Frost and Lish, 1975). Another agent evaluated as a potential countermeasure for radiation induced oxidative stress is a soybean-derived protease inhibitor known as the Bowman-Birk inhibitor (BBI), which has been developed in the form of BBI Concentrate (BBIC) for cancer prevention and human trials, as reviewed previously (Kennedy, 1993, 1998a, 1998b, 2005). Both BBI and BBIC have been shown to have antioxidant properties (Kennedy, 1998a) and BBI has been utilized as a radioprotective agent (Dittmann et al., 1995, 1998a, 1998b, 2000, 2001, 2003; Kennedy et al., 1996; Gueven et al., 1998a, 1998b).

Using the DCF fluorometric assay method previously adapted for measurement of radiation induced oxidative stress in cultured cells (Wan et al., 2003, 2005a), we performed experiments with X-rays, γ -rays, protons and HZE particles to evaluate the protective effects of antioxidants against radiation induced oxidative stress and found NAC, ascorbic acid, α -lipoic acid and SeM to be highly effective in preventing radiation induced oxidative stress, whereas

coenzyme Q10 and vitamin E succinate were only weakly effective in preventing radiation induced oxidative stress in cultured cells (Kennedy et al., 2004; Wan et al., 2006). Based on the results of the DCF fluorometric assay experiments, these antioxidants were selected as a combination for further studies *in vitro* and *in vivo*.

The ability of the antioxidant combination and BBIC to prevent radiation induced oxidative stress *in vivo* was evaluated in Sprague-Dawley rats and CBA mice irradiated with γ -ray, proton or ^{56}Fe ion radiation using the total antioxidant status (TAS) in serum or plasma as the biological endpoint (Kennedy et al., 2004, 2007; Guan et al., 2004, 2006). In the rat studies, the serum or plasma level of total antioxidants was found to be decreased after exposure to γ -ray or 1-GeV/n ^{56}Fe ion radiation, and the decrease was alleviated or completely prevented in the animals fed with a diet supplemented with SeM (12 $\mu\text{g/g}$ diet) alone or in combination with sodium ascorbate (19 $\mu\text{g/g}$ diet), NAC (51 $\mu\text{g/g}$ diet), α -lipoic acid, reduced form (100 $\mu\text{g/g}$ diet), vitamin E succinate (8.6 $\mu\text{g/g}$ diet) and coenzyme Q10 (51 $\mu\text{g/g}$ diet) (Kennedy et al., 2004; Guan et al., 2004). In the mouse studies, the plasma TAS also decreased significantly after exposure to 0.5 Gy of 1-GeV/n ^{56}Fe ion radiation or 3 Gy of 1-GeV proton or γ -ray radiation, and the decrease in plasma TAS was alleviated or prevented completely by diet supplementation with BBIC (10 mg/g diet), SeM (0.14 $\mu\text{g/g}$ diet), or a combination of L-SeM (0.14 $\mu\text{g/g}$ diet), sodium ascorbate (17.14 $\mu\text{g/g}$ diet), NAC (51.43 $\mu\text{g/g}$ diet), α -lipoic acid (102.86 $\mu\text{g/g}$ diet), vitamin E succinate (8.57 $\mu\text{g/g}$ diet) with or without coenzyme Q10 (51.43 $\mu\text{g/g}$ diet) (Guan et al., 2006; Kennedy et al., 2007). These results indicate that BBIC, SeM and the antioxidant combinations are potentially useful as countermeasures against space radiation-induced oxidative stress and subsequent adverse biological effects, which could arise from the increased oxidative stress in irradiated subjects.

5.2. Antioxidant protection against radiation induced cell death and transformation *in vitro*

The protective effects of the antioxidants, BBI and BBIC against radiation induced cell death have been evaluated *in vitro* using the clonogenic survival of cultured MCF10 cells or HTori-3 cells as the biological endpoints (Kennedy et al., 2004, 2006). Irradiation with 5-GeV/n ^{56}Fe ions resulted in a dose-dependent decrease in the clonogenic survival of the MCF10 cells, which was attenuated by treatment with SeM alone or in combination with ascorbic acid, coenzyme Q10 and vitamin E succinate with an estimated dose modifying factor (DMF) of 1.2, 1.4, 2.2 and 2.2, respectively, for BBI, BBIC, SeM alone or in combination with other antioxidants (Kennedy et al., 2006). The identical DMF values for treatments with SeM alone or in combination with other antioxidants indicate that these antioxidant combinations were not more effective than the SeM treatment alone under the experimental conditions utilized. These results do not rule out the possibility that the antioxidant combinations might have provided better protection than SeM treatment alone under other experimental conditions or for other biological endpoints, however, since a combination of antioxidants with different biochemical properties and action mechanisms is likely to provide better protection for different molecular targets during and after irradiation. For examples, cationic thiols are much more effective than anionic thiols in protecting DNA against radiation damage (Fahey, 1988; Prise et al., 1995), and lipophilic antioxidants, such as vitamin E, are effective protectors of biomembranes (Wolf et al., 1998; Gencel et al., 2010), whereas hydrophilic antioxidants are more effective in protecting soluble proteins and enzymes in the aqueous environment of cells (Boldyrev, 2005).

The protective effects of the antioxidants, BBI and BBIC, against proton and HZE particle radiation induced cell transformation were

determined in HTori-3 cells by the soft agar colony formation assay, which measures the capability of HTori-3 cells to grow in an anchorage-independent manner. The results indicate that treatment of the cells with BBI, BBIC, SeM alone or in combination with ascorbic acid, coenzyme Q10 and vitamin E succinate prevented proton and HZE particle radiation induced HTori-3 cell transformation *in vitro* (Kennedy et al., 2004, 2006). In experiments performed with γ -ray radiation, treatment with 5 μM SeM before, during and/or as late as 7 days after the radiation exposure brought the anchorage-independent colony formation efficiency down to levels that were not significantly different from the sham radiation controls (Ware et al., 2011).

5.3. Antioxidant protection against space radiation induced mortality

It is well known that the hematopoietic system is highly sensitive to total body irradiation (TBI) and the fate of hematopoietic cells after TBI may determine the survival or death of irradiated subjects (Mettler and Voelz, 2002; Koenig et al., 2005). Thus, we have evaluated the effects of antioxidants in mice using the 30-day survival level and hematopoietic cell counts as the biological endpoints. Dietary supplementation with an antioxidant combination consisting of SeM (0.06 $\mu\text{g/g}$ diet), α -lipoic acid (85.7 $\mu\text{g/g}$ diet), NAC (171.4 $\mu\text{g/g}$ diet), sodium ascorbate (142.8 $\mu\text{g/g}$ diet) and vitamin E succinate (71.4 $\mu\text{g/g}$ diet) significantly improved the 30-day survival of the mice irradiated with 8 Gy of X-rays (Wambi et al., 2008) or 5.9 Gy of protons (Wambi et al., 2009). However, no significant improvement was observed for the mice irradiated with protons at higher radiation doses (6.8 or 7.2 Gy) (Wambi et al., 2009). It is expected that the higher proton radiation doses used in these studies might have caused damage that was beyond mitigation by the antioxidant treatment. In both the proton and X-ray radiation experiments, antioxidants were more protective when the antioxidant treatment was initiated 2 hours after radiation exposure as compared to the antioxidant treatment initiated 7 days prior to radiation exposure (Wambi et al., 2008, 2009). These results were confirmed by other investigators, who also showed that the antioxidant combination had a better protective effect on radiation induced lethality when started 7 days post-irradiation than it did when it was started 2 hours post-irradiation (Brown et al., 2010). The findings that antioxidant treatment leads to better survival when applied post-irradiation might result from an adaptive response to radiation exposure, which has been reviewed recently (Matsumoto et al., 2007, 2011). The ability of the antioxidants to improve radiation survival even when the antioxidant treatment was initiated after the radiation exposure suggests that antioxidants can be a feasible countermeasure for radiation exposure associated with space travel, radiation accidents or terrorist attacks in which radiation exposure could occur without much advanced warning.

The antioxidant treatment significantly attenuated the radiation effects on peripheral hematopoietic cell counts in the mice irradiated with 1 or 8 Gy of X-rays (Wambi et al., 2008) or 1 or 7.2 Gy of 1-GeV protons (Wambi et al., 2009). The antioxidant treatment was also shown to improve the recovery of the bone marrow cell counts in mice irradiated with γ -rays (Wambi et al., 2008) or 1-GeV protons (Wambi et al., 2009). Thus, antioxidants appear to be effective for protection of hematopoietic cells against the adverse effects of either photon or proton radiation. The ability of antioxidants to prevent the radiation caused loss of circulating neutrophils, also called PMNs, are illustrated in Fig. 8, which shows that the mice maintained on an antioxidant diet and exposed to an 8 Gy dose of radiation experienced a drop in the levels of PMNs/neutrophils of a magnitude comparable to the effects observed for mice exposed to a 1 Gy dose of radiation and maintained on the normal diets. The mice maintained

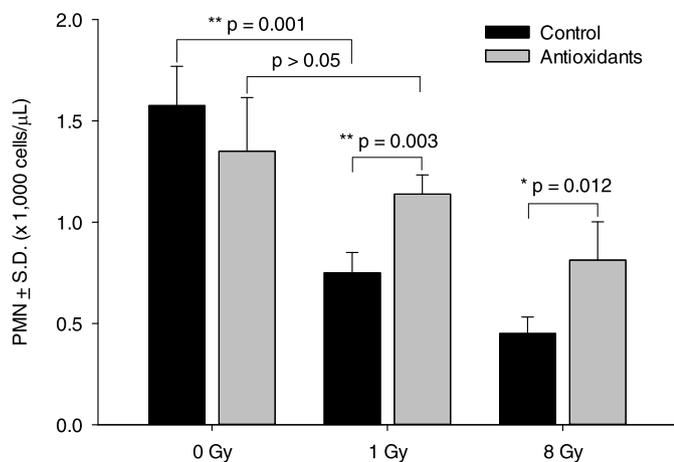


Fig. 8. Effects of radiation on polymorphonuclear (PMN)/neutrophil cell counts in mice treated with antioxidants. In this experiment, animals were maintained on the control diet or antioxidant supplemented diet and irradiated with x-rays at doses of 1 or 8 Gy, which significantly decreased the numbers of circulating PMN cells (neutrophils) to a greater extent in mice maintained on the control diet than in mice maintained on the antioxidant supplemented diet. The magnitude of PMN cell loss in mice maintained on the antioxidant diets and exposed to 8 Gy of radiation was comparable to that observed in the mice maintained on the control diet and exposed to a 1 Gy dose of radiation. The decrease in the PMN cell counts in mice maintained on the antioxidant supplemented diet and exposed to a 1 Gy dose of radiation was not significantly different from that in the control animals. These results indicate major beneficial effects of antioxidants on the survival of circulating PMN cells/neutrophils following the radiation exposure. [These data are representative of those published previously (Wambi et al., 2008).]

on the antioxidant diets and exposed to a 1 Gy dose of radiation had PMN/neutrophil cell counts that were not significantly different from those in the control animals. Thus, it is concluded that the antioxidant diet had highly significant beneficial effects on PMN/neutrophil cell counts in irradiated animals.

5.4. Antioxidant protection against space radiation induced cataracts

An increased rate of cataract formation has been observed in astronauts (Cucinotta et al., 2001; Rastegar et al., 2002), which has been attributed to the increased exposure to cosmic radiation during space travel. To investigate the ability of BBIC and antioxidants to reduce the formation and severity of cataracts related to space radiation exposure, mice were exposed to 1-GeV proton or 1-GeV/n ^{56}Fe ion radiation and fed with a control diet or diets supplemented with BBIC or the antioxidant combination containing SeM, NAC, ascorbic acid, coenzyme Q10, α -lipoic acid and vitamin E succinate before and for 2 years after the radiation exposure (Davis et al., 2010). Lenses were harvested approximately 2 years after the radiation exposure for evaluation of the lens opacifications. The results showed that treatment with BBIC or the antioxidant combination decreased the prevalence and severity of the lens opacifications in the mice irradiated with 3 Gy of 1-GeV proton or 0.5 Gy of 1-GeV/n ^{56}Fe ion radiation, although statistical significance was only achieved for the ^{56}Fe ion irradiated mice, possibly due to the higher proton radiation dose (3 Gy) that might have exceeded the protective capacity of the antioxidant combination or BBIC. These results indicate that BBIC and the antioxidant combination could be useful for protecting astronauts against space radiation-induced cataracts during or after long-term manned space missions.

5.5. Antioxidant prevention of space radiation induced cancer

Radiation induced malignancy is a particularly important risk associated with extended space travel. In a 2-year study performed with CBA/JCR HSD mice irradiated with 3 Gy of 1-GeV protons

or 0.5 Gy of 1-GeV/n ^{56}Fe ions, treatment with the antioxidant combination or BBIC decreased the fractions of animals with malignant lymphoma to levels that were not significantly different from the baseline level (Kennedy et al., 2008). The treatment with the antioxidant combination or BBIC also prevented the increase in the fractions of mice with premalignant or malignant lesions of myeloid origin after the proton irradiation and the incidence rate of rare tumors (which included Harderian gland tumors) after the proton or ^{56}Fe ion radiation exposures (Kennedy et al., 2008, 2011). From these experiments, it was concluded that antioxidants have a major protective effect against space radiation induced carcinogenesis *in vivo* (Kennedy et al., 2008, 2011). In these studies, a major protective effect resulted from the ability of the antioxidants to prevent the early stage neoplastic growths from growing into fully developed, malignant tumors. Other studies suggest that anticarcinogenic agents can be added at late times following carcinogen exposure, in both *in vitro* and *in vivo* systems, and still have a suppressive effect on the carcinogenic process (Kennedy, 1998b). The results of the studies on radiation induced carcinogenesis suggest that antioxidant and BBIC supplements could be useful for the prevention of malignancies and other neoplastic lesions developing as a result of exposure to space radiation. It has been concluded from these studies that antioxidants have a major protective effect against radiation induced carcinogenesis, and are effective even when added at late times during the carcinogenic process.

5.6. Mechanism(s) for antioxidants as radiation countermeasures

To study the mechanisms for the radioprotection by the antioxidant treatment, we have examined the expression of the ATR gene, which is one of the central components of the DNA damage response pathway (Durocher and Jackson, 2001), and the CHK2 gene, which is a cell cycle checkpoint regulator and putative tumor suppressor (Xu et al., 2001; Hirao et al., 2002), in HTori-3 cells irradiated with 0.4 Gy of 5-GeV/n ^{56}Fe ions with or without SeM (5 μM) treatment initiated 24 hours prior to the radiation exposure. The results indicate that the ATR mRNA level was increased by 42% with the SeM treatment alone and increased by 94% with the SeM treatment and radiation exposure (Kennedy et al., 2004). In the same study, SeM treatment alone did not significantly affect the CHK2 mRNA level, but the combined treatment with SeM and ^{56}Fe ion radiation increased the level of CHK2 mRNA by 99%. The up-regulation of ATR and CHK2 gene expression observed in the irradiated HTori-3 cells may prevent the cells from going through mitosis until the damage is repaired, thereby preventing the radiation damage from being fixed and leading to mutations and/or malignant transformation.

In ICR mice irradiated with 6.4 Gy of 1-GeV protons or 7.0 Gy of γ -rays, 15 genes belonging to the class of "apoptosis regulator activity" were differentially expressed in the spleen of mice fed an antioxidant supplemented diet as compared with mice fed with the control diet, and the antioxidant treatment inhibited apoptosis in the white pulp of the spleen following γ -ray irradiation, possibly by altering IL-6 signaling and by blocking the expression of the prokineticin PROK2, the ligand to the G protein-coupled receptors PROKR1 and PROKR2 (Finnberg et al., 2013), which are involved in a number of pathophysiological processes. In ICR mice irradiated with 1 or 8 Gy of X-rays, bcl-2 gene expression was found to be decreased, whereas bax, caspase 7, caspase 9 and TGF- β 1 gene expression was increased at 4 or 24 hours after irradiation. Dietary supplementation with antioxidants attenuated the radiation effects on bax, caspase 7, caspase 9 and TGF- β 1 gene expression, but increased bcl-2 gene expression by 10 fold at 24 hours after irradiation (Wambi et al., 2008). The abrogated proapoptosis (bax and caspase 9) gene expression and the increased

anti-apoptosis (bcl-2) gene expression observed in the irradiated animals treated with antioxidants have implicated apoptosis as a key process modulated by antioxidants to attenuate the effects of radiation on the hematopoietic system and animal survival (Wambi et al., 2008). The anti-apoptotic effects of selenium have also been reported in normal human or mouse fibroblasts (Seo et al., 2002) and primary human keratinocytes (Rafferty et al., 2003) irradiated with ultraviolet irradiation. The anti-apoptotic effect of selenium observed in normal animals or tissues after irradiation is in contrast to the pro-apoptotic effect of selenium observed in malignant cell lines or tissues (Husbeck et al., 2005; Fischer et al., 2006; Zhao et al., 2006). The differential effects of selenium on apoptosis in normal and malignant cells/tissues suggest the possibility that selenium may protect normal tissues against radiation damage without the unintended consequence of suppressing radiation induced apoptosis in malignant cells or tissues. The different effects of antioxidants in normal cells, as compared to their effects in malignant cells, or those in different stages of carcinogenesis, have been discussed elsewhere (Brash and Havre, 2002).

In a separate study performed with cultured HTori-3 cells exposed to low doses (0.1 and 0.2 Gy) of 1-GeV/n ^{56}Fe ion radiation, treatment with 5 μM SeM in the medium for 24 hours prior to irradiation profoundly affected the radiation induced alterations in gene expression (Stewart et al., 2006). The exposure to 0.1 and 0.2 Gy of ^{56}Fe ion radiation induced significant differential expression of 196 and 610 genes, respectively, and the differential expression of 39% to 55% of these genes was abolished by the SeM treatment (Stewart et al., 2007). Genes and functional pathways that were significantly up- or down-regulated by the ^{56}Fe ion irradiation in the absence, but not in presence, of SeM treatment have been summarized previously (Stewart et al., 2006, 2007). Of particular interest was a cluster of chemokine and cytokine genes, e.g., CXCL1, CXCL2, IL6, IL11, IL8, IL24 and TGF β 2, which showed increased expression after irradiation with 0.1 Gy of 1-GeV/n ^{56}Fe ions in the absence, but not in the presence, of 5 μM SeM in the medium before and during the radiation exposure (Sanzari et al., 2009). It is also noteworthy that SeM has been shown to reduce space radiation induced effects by mitigating stress-related signaling pathways and downregulating certain genes associated with cell adhesion (Nuth and Kennedy, 2013). These results suggest that SeM is potentially useful as a countermeasure to prevent some of the acute inflammatory/immune responses induced by low-dose HZE particle radiation.

5.7. Granulocyte colony-stimulating factors as countermeasures

In addition to the antioxidants, two forms of granulocyte colony-stimulating factors (G-CSFs), filgrastim and pegfilgrastim, were evaluated as countermeasures using the neutrophil count in ICR mice irradiated with γ -rays or SPE-like protons as the biological endpoint. The results demonstrated that exposure to SPE-like proton radiation or γ -ray radiation at doses up to 2 Gy significantly decreased circulating neutrophil counts in a dose and time dependent manner, which was prevented by treatment with either form of G-CSF evaluated in the study (Romero-Weaver et al., 2013a). These results indicate that both forms of G-CSFs could be a potential countermeasure for the reduced number of neutrophils in irradiated animals, although pegfilgrastim appears to be superior since its stimulatory effect on the neutrophil count was more pronounced and lasted longer than that of filgrastim.

In mouse studies using G-CSF (Neulasta) as a countermeasure for bacterial challenge toxicity in animals exposed to SPE radiation along with HS, Neulasta treatment was shown to reduce morbidity from 80–90% to 20–30% in γ -ray or proton irradiated C3H/HeN mice exposed to HS and challenged with *Pseudomonas aeruginosa* bacteria (Drew Weissman and colleagues, Unpublished data). In an

experiment performed with ferrets (aged 12–15 weeks), that were exposed to a 2 Gy total body dose of γ -ray radiation with subcutaneous injection of phosphate buffered saline (PBS) or Neulasta (0.1 mg/kg) on days 1, 4, and 7 post-irradiation, peripheral blood was collected from each animal on day 0 (prior to radiation exposure), and on days 1, 4, 7, and 13 post-irradiation for analysis. The results demonstrate that the Neulasta treatment of irradiated ferrets could lead to a significant increase in neutrophil counts (Krigsfeld, G.S., Sanzari, J.K. and Kennedy, A.R., Unpublished data). In a similar experiment performed with pigs (aged 12–15 weeks) that were exposed to a 2 Gy total body dose of SPE-like proton radiation and subcutaneous injections of PBS or Neulasta (0.1 mg/kg) on days 4, 7, and 10 post-irradiation, peripheral blood was collected from each animal on day 0 (prior to radiation exposure), and on days 1, 7, 10, 13, and 30 post-radiation for analysis. The results indicated that the proton radiation exposure led to statistically significant decreases in neutrophil counts at days 7, 10 and 13 post-irradiation in the irradiated animals injected with PBS; however, in irradiated pigs treated with Neulasta, the neutrophil counts were never decreased to below the baseline levels (Sanzari, J.K., Krigsfeld, G.S., Shuman, A.L. and Kennedy, A.R., Unpublished data). The overall conclusion from the studies in mice, ferrets and pigs is that Neulasta can increase the number of circulating neutrophils in three different species of animals evaluated.

5.8. SI-Wu-Tang or fructose as countermeasures to increase neutrophil counts in mice exposed to SPE or γ -ray radiation

A Chinese formula SI-Wu-Tang (SWT, 四物汤) is currently given to radiation and chemotherapy cancer patients in China to mitigate the adverse effects of cancer therapy on the numbers of circulating blood cells. One of the major ingredients in SI-Wu-Tang is fructose, as reviewed (Romero-Weaver et al., 2014). We have performed studies in mice to determine whether these compounds in traditional Chinese medicine were able to mitigate reduced circulating blood cell counts following γ -ray or SPE-like proton radiation. The main conclusions of the studies were as follows: 1) SWT and fructose were both capable of increasing the number of circulating lymphocytes in γ -ray or SPE proton irradiated mice, and 2) fructose was more effective than SWT in increasing the number of circulating lymphocytes in γ -ray or proton irradiated mice (Romero-Weaver et al., 2014). These results are important since there are essentially no countermeasures for lymphocyte loss following radiation exposure. Lymphocytes are among the most sensitive cells in the body to radiation exposure (Sanzari et al., 2013d, 2013e, 2013b; Neff and Cassen, 1968), and a reduced lymphocyte count in the circulation at post-irradiation times is expected to result in greater susceptibility to infections. The fact that both SWT and fructose can be given orally makes them attractive for use during space travel.

5.9. Antibiotics as countermeasures for bacterial toxicity in mice exposed to SPE radiation and HS

Enrofloxacin, an antibiotic for veterinary use, was studied for its effectiveness as a countermeasure in experiments using the bacterial challenge model in mice. Enrofloxacin is available in an oral form but the mice in these experiments were treated with subcutaneous drug. Control, irradiated (2 Gy), hindlimb suspended, and irradiated and hindlimb suspended mice were treated with *Pseudomonas aeruginosa* bacteria at a dose that can be cleared by untreated animals or, in later experiments, with a dose of bacteria that leads to morbidity in approximately 50% of untreated mice. With both challenge doses, the institution of enrofloxacin at the time of bacterial challenge reduced morbidity from 80–100% to 0%. This demonstrated the complete effectiveness of prophylactic

antibiotic treatment in the protection of irradiated and hindlimb suspended mice against bacterial toxicity with the potential to lead to morbidity/death (Drew Weissman, Unpublished data).

While the effectiveness of the antibiotic countermeasure cannot be surpassed, it does carry a number of potential adverse effects, especially, if it will need to be used multiple times, including the generation of antibiotic resistant bacteria. Thus, it is believed that alternative countermeasures for bacterial toxicity, with different mechanisms of action than those of antibiotics, will be useful for space travel in future exploration class missions.

5.10. Countermeasures for radiation induced emesis in ferrets

In studies performed with ferrets, 5-HT₃ receptor antagonists, such as Zofran, have been shown to prevent or ameliorate vomiting and retching in ferrets following proton radiation exposure (King et al., 1999). As Zofran is maintained on the ISS for nausea and vomiting, it is the recommended 5-HT₃ receptor antagonist to prevent or mitigate radiation induced nausea and vomiting during space travel.

5.11. Countermeasures for altered bleeding times in ferrets after SPE radiation exposure

It is well established that endotoxin (lipid A portion) released by Gram negative bacteria activates certain factors of the intrinsic coagulation cascade, such as Factor XII, which in turn initiates fibrin formation and increases PT/aPTT values, thereby increasing the risk of DIC development. To counteract the factor deficiencies, BeneFIX (recombinant Factor IX) was evaluated as a countermeasure for the proton radiation induced activation of the coagulation cascade. Treatment with BeneFIX reduced the clotting times in irradiated ferrets back to levels that were essentially equivalent to those of the non-irradiated control ferrets (Krigsfeld et al., 2013). Since treatment with BeneFIX increases the concentration of Factor IX, a factor depleted post-irradiation, BeneFIX could have beneficial effects on coagulation when administered after the radiation exposure.

Phytonadione (vitamin K) is essential for post-translational modification of a glutamate to a carboxylated-glutamate that is necessary for Factor II, VII, IX, and X (Furie and Furie, 1988). It was also evaluated as a countermeasure for activation of the proton radiation induced coagulation cascade. The results indicate that phytonadione had a minor beneficial effect on PT values in the proton irradiated ferrets, but did not affect the aPTT values (Krigsfeld et al., 2013).

Treatment of DIC with blood clotting factors or other components of plasma is a topic that has been reviewed previously (Levi et al., 2009). We have demonstrated that SPE-like proton radiation led to hypocoagulability by activating the clotting cascade that consumes factors involved in coagulation. SPE-like proton radiation can also cause leucopenia and severe lymphocytopenia, which, in combination with the effects of the SPE-proton radiation on hemostasis, could have major health consequences in irradiated subjects. Our studies have shown that BeneFIX can serve as a potential countermeasure for the increased bleeding times in ferrets caused by exposure to SPE-like proton radiation.

5.12. Corticosteroid as a countermeasure for radiation induced pneumonitis and pneumonopathy in pigs

Several pigs exposed to simulated SPE radiation developed symptomatic, radiation-associated pneumonopathy that radiographically involved all lung fields but was worse in the pleura and apices where the radiation dose was found to be highest (Wilson

et al., 2011); many of the pigs with this condition developed non-productive coughs. Thoracic radiographs and diagnostic CT scans were performed, and the CT findings were consistent with an acute lung injury concurrent with chronic bronchial changes and volume loss. Differential diagnoses for these findings were radiation induced lung injury or atypical infectious bronchopneumonia. To help differentiate between these possibilities, sequential antibiotic therapy, which included atypical (e.g., mycoplasma), Gram-positive and Gram-negative coverage, was initiated. After two weeks of therapy, it was concluded that antibiotics were not effective treatments for the condition. Corticosteroid therapy then began and the pig symptoms improved rapidly, with dramatic improvement and resolution of imaging abnormalities observed within one month. It was concluded that corticosteroids were effective treatments for the pneumonopathy and/or pneumonitis that developed in the pigs exposed to SPE-like radiation (Wilson et al., 2011).

5.13. Mometasone as a countermeasure for SPE proton radiation induced skin lesions in pigs

Pigs were exposed to either a 5 Gy or 10 Gy dose of SPE-like electron irradiation, and seven types of creams were applied to the trunk of the animal in 1 inch² patches covered with Tegaderm dressing consecutively for 14 days immediately after the radiation exposure. The only cream that appeared to mitigate the radiation-induced initial hyperpigmentation was the cream that contained corticosteroids (mometasone cream [Elecon]), as compared to the other creams, which were water-based emollients, a platelet growth factor containing cream, a triple antibiotic containing cream, or the untreated area that was not exposed to any cream other than the Tegaderm dressing. At 14 days post-irradiation, biopsies were performed that revealed decreased melanosomes, necrotic keratinocytes and melanin deposition in the areas of irradiated skin treated with mometasone compared to the untreated irradiated skin. Thus, it was concluded from these studies that topical application of steroids mitigate skin toxicity produced by exposure to SPE-like proton radiation (Cengel, K.A. and Sanzari, J.K., Unpublished data).

5.14. Transparent film dressing for protection against proton therapy induced skin reactions in humans

Patients undergoing proton therapy for prostate cancer frequently develop radiation dermatitis. It has been reported that two prostate cancer patients undergoing proton cancer therapy at the University of Pennsylvania had radiation dermatitis that appears to have been substantially diminished by the presence of transparent film dressings (Beekley stickers) (Whaley et al., 2013). In these studies, small circular (2.5 cm diameter) transparent adhesive markers were placed on their skin to assist with daily alignment in these patients treated with a total proton dose of 79.2 Gy in 1.8 Gy fractions, using two opposed lateral beams daily. It was observed that the covered areas of the skin exhibited considerably diminished radiation dermatitis compared to the uncovered areas of the skin, and this difference persisted for at least one month after the therapy period ended. A phantom dosimetric study was performed to evaluate the impact of the transparent film dressing on a beam's SOBP, and the results indicated no gross dosimetric effect. Thus, the transparent adhesive markers appear to have attenuated radiation dermatitis in these two patients without affecting the SOBP. It is hoped that this finding can improve proton-related radiation dermatitis in other types of treatments for cancer as well (i.e., in other conditions in which proton radiation exposure could lead to radiation dermatitis).

6. Discussion

In recent years, we have been engaged in *in vitro* and animal studies on acute biological effects of the types of radiation at the energies, doses and dose-rates relevant to space travel. Three species of animals, i.e., ferrets, pigs and mice, were used in our studies to evaluate the effects of radiation on various biological endpoints including survival, cancer development, hematopoietic cell counts, emesis, blood coagulation, CNS endpoints (which included social exploration, submaximal exercise treadmill, and locomotor activity), cataract development, oxidative stress, gastrointestinal tract bacterial translocation, immune activation, and gene expression associated with programmed cell death and ECM remodeling.

For 30-day survival, 1-GeV/n proton radiation appeared to be more lethal than X-rays to mice (Wambi et al., 2008, 2009), but SPE-like proton radiation was comparable to γ -rays for ferrets (Krigsfeld et al., 2014). Ferrets are considerably more sensitive than mice to the lethal effects of radiation exposure. In experiments with mice irradiated at lower doses of protons (3 Gy) or HZE particles (0.5 Gy), acute effects of the radiation doses were not observed over a 30 day experimental period, but the long-term survival in the irradiated mice was reduced significantly by the radiation exposure, and the decreased long-term survival was accompanied by a significant increase in the rate of development of malignant lymphomas and Harderian gland tumors, as well as the fractions of animals with malignant lymphoma or rare tumors (Kennedy et al., 2008, 2011).

With respect to peripheral WBC and lymphocyte counts, the effects of proton irradiation given as a homogeneous dose to the mice is not affected by the dose fractionation, dose-rates or proton energy in the ranges evaluated. In contrast, simulated hypogravity brought about by PWS was shown to potentiate splenic lymphocytes to the cell killing effects of radiation (Sanzari et al., 2011b). A number of other studies indicated that SPE radiation and simulated microgravity produced by HS led to synergistic adverse effects on hematopoietic and immune cell functions, including bacterial containment in the GI tract (Zhou et al., 2012), T-cell activation (Sanzari et al., 2011b, 2013a), and death from a bacterial challenge at a sub-lethal dose (Li et al., 2014).

For emesis in ferrets, the risk of SPE radiation-induced vomiting is low and may reach statistical significance only when the radiation dose reaches 1 Gy or higher. The ED₁₀ and ED₅₀ values estimated for the fraction of animals that retched or vomited after proton irradiation at the high dose rate (0.5 Gy/minute) were lower than the lower limits of the respective 95% confidence intervals established for γ -rays, suggesting that high dose rate proton irradiation was more effective than high dose rate γ -ray irradiation in inducing retching and vomiting. There was a large sparing effect observed at the low dose rates expected for SPE radiation, such that the results for vomiting and retching in response to low dose-rate exposure to SPE radiation were not statistically significant when compared to control animals. The trend, however, in the experiments performed at the low dose rate appeared to indicate more retching and vomiting episodes in ferrets irradiated with protons than in the animals exposed to γ -rays at the same dose and dose rate (Sanzari et al., 2013c).

The results from some of the studies described here indicated that proton radiation had considerably more severe adverse effects compared to those produced by comparable doses of the reference radiations used, which include x-rays, γ -rays and electrons. These include studies on mouse survival after irradiation with protons and x-rays (Wambi et al., 2008, 2009), on ferret hematopoietic cell counts after exposure to SPE protons and γ -rays (Sanzari et al., 2013d), on ferret retching and vomiting induced by SPE protons and γ -rays (Sanzari et al., 2013c), on blood clotting times

in ferrets exposed to SPE protons and γ -radiation (Krigsfeld et al., 2012) and on peripheral hematopoietic cell (WBC, lymphocyte and neutrophil) counts in minipigs exposed to simulated proton SPE or simulated electron SPE radiation (6 + 12 MeV electrons), which resulted in a comparable dose distribution to the proton SPE radiation expected in an SPE, especially at the low end of the radiation dose range evaluated (Sanzari et al., 2013b). The results of other studies described here suggest that the effects of proton radiation were comparable to those of the reference radiations evaluated. These studies include studies on the lack of bacterial containment in the GI tract from exposure to γ -rays or SPE proton radiation in mice (Ni et al., 2011), on fatigue in mice after irradiation with γ -rays or protons (York et al., 2012a; York, 2012), on the skin changes resulting from exposure to SPE proton or electron radiation (Wilson et al., 2011), on the development of DIC in irradiated ferrets (Krigsfeld et al., 2014) and on mortality of mice exposed to radiation alone or radiation with exposure to microgravity, along with a bacterial challenge (Drew Weissman and colleagues, Unpublished data). Overall, the differences in the effects of the proton and reference radiation were relatively small or limited to the lower end of the dose range (~0.5 Gy) evaluated.

The mechanism(s) for mortality in irradiated mammals is not well understood, although numerous hypotheses have been proposed. At the lowest total body radiation doses leading to mortality, death occurs from the hematopoietic syndrome, which is believed to result from the cell killing effects of radiation in the bone marrow that lead to low numbers of circulating blood cells and the resultant hematopoietic symptoms, such as infection and bleeding due to the loss of leukocytes and platelets. Over the last half century, the radiation dose required to kill half of the irradiated subjects, known as the LD₅₀, has been used as a parameter of radiation sensitivity for comparisons among various mammalian species. It is well-known that the LD₅₀ value is highly variable for different mammalian species; however, the bone marrow cell sensitivity to ionizing radiation is remarkably similar among different species, strains and individuals (Bond and Robinson, 1967; Morris and Jones, 1988). These results suggest that the lethal effects of radiation on bone marrow and hematopoietic cells may not be the primary mechanism for radiation exposure related death. Our studies in ferrets irradiated with SPE-like protons have suggested that radiation induced activation of the coagulation cascade may result in DIC, which could be a major mechanism by which relatively low doses of radiation lead to mortality (Krigsfeld et al., 2014). The human LD₅₀ values for radiation induced death are imprecise because there have been relatively few cases in which human subjects were exposed to radiation at doses near the LD₅₀, especially in the last half century. The estimated human LD₅₀ value for ionizing radiation ranges from 3 to 4 Gy for young adults, without medical intervention to 2 to 3 Gy for the very young or the old (Hall and Giaccia, 2006). Remarkably different LD₅₀ values have been reported for different species (Morris and Jones, 1988; Hall and Giaccia, 2006; Harding, 1995) with ferrets as the most sensitive mammalian species (Krigsfeld et al., 2014; Harding, 1995), closely followed by dogs and pigs (Morris and Jones, 1988). The LD₅₀ in Gottingen pigs is as low as 1.8 Gy and widespread hemorrhaging is observed at death of the irradiated Gottingen pigs (Moroni et al., 2011a), with some evidence of DIC at doses near the LD₅₀, such as the rapid onset of systemic inflammation (C-reactive protein, fibrinogen) and multi-organ dysfunction (Moroni et al., 2011b). Similarly, dogs exhibit hemorrhagic diathesis at doses near the LD₅₀, and die with signs resembling DIC (Andersen, 1957; Winchell et al., 1964). While DIC has not been diagnosed as a cause of radiation induced death in the pig or dog studies or in irradiated human populations, a hallmark of DIC, i.e. hemorrhage, at death has been frequently

observed in irradiated mammals, including humans. There is extensive evidence that widespread hemorrhages occurred in the Hiroshima and Nagasaki atomic bomb casualties, even in the relatively low radiation dose groups (Liebow et al., 1949), with the estimated LD₅₀ values of approximately 2.5 (Lushbaugh, 1969; Fujita et al., 1991). Other information about hemorrhaging in humans after irradiation comes from accidental exposures in Norway (Reitan et al., 1990) and Brazil (Valverde et al., 1990; Rosenthal et al., 1991) in which several people were accidentally exposed to whole body irradiation at doses near the human LD₅₀ and widespread hemorrhages were observed.

A reduction in the number of platelets can result in hemorrhaging and death; however, our studies suggest that the blood clotting abnormalities in irradiated ferrets is not caused by a reduction in the number of platelets (Krigsfeld and Kennedy, 2012; Krigsfeld et al., 2013, 2014). A similar phenomenon has been reported in irradiated dogs in which the platelet counts are not depressed in dogs at a “preterminal” stage in which bleeding abnormalities are observed, although the platelet counts are decreased greatly in irradiated dogs with full-blown DIC (Winchell et al., 1964). In atom bomb casualties, widespread hemorrhages were observed in people before the level of platelet counts had fallen to levels expected to cause hemorrhage (Liebow et al., 1949). Furthermore, while platelet injections and other blood clotting factors can have beneficial effects on hemorrhaging, platelet infusions do not prevent all deaths from the radiation exposure (Lorenz and Congdon, 1954).

Hemorrhaging and signs of DIC have been frequently reported in higher mammalian species, such as dogs and pigs; however, hemorrhaging has not been reported in mice at doses near the LD₅₀. Mice are the animals that are most often used in radiobiology studies. Bacteremia is the leading cause of death in mice (Lorenz and Congdon, 1954; Miller et al., 1951), whereas hemorrhage is thought to be the major cause of death in dogs, rabbits, guinea pigs (Lorenz and Congdon, 1954) or pigs (Eisele and West, 1973) after irradiation at doses near the LD₅₀ dose for each species. The pathophysiology of the hematopoietic syndrome in pigs is thought to be similar to that observed in humans (Moroni et al., 2011a). Such differences suggest that we need new ways of thinking about mechanisms for death after irradiation at doses near the LD₅₀ level. Since the human LD₅₀ is closer to the LD₅₀ of ferrets, dogs and pigs than to the LD₅₀ of mice, species such as ferrets, dogs and pigs are likely to be better animal models than mice for evaluating the effects of radiation and radiation countermeasures on bleeding, coagulation cascade activation and DIC risks for humans.

It is noteworthy that the LD₅₀ values for these larger mammals are considerably lower than those observed in various strains of mice (Morris and Jones, 1988). Examples of some published LD₅₀ values for mammals are as follows: Ferrets – 1.5 Gy (Krigsfeld et al., 2014), Gottingen pigs – 1.8 Gy (Moroni et al., 2011a), pigs – average value – 2.6 Gy (Morris and Jones, 1988), dogs – average value – 2.6 Gy (Morris and Jones, 1988), monkeys – 5.07 Gy (Eldred and Trowbridge, 1954), mice – average value – 8.16 Gy (Morris and Jones, 1988). Using published data, we have compared the percentage of animals exhibiting hemorrhage at death (from exposure to radiation doses near the LD₅₀ level) as a function of the LD₅₀ of the species and observed an excellent correlation between the percent of animals developing “DIC” (from hemorrhaging at death, the hallmark of DIC) and the LD₅₀ value for the species/strain (Krigsfeld, G.S. and Kennedy, A.R., Unpublished data). These figures for animal hemorrhaging at death range from 100% of ferrets – to 0% of the mice (as mice irradiated at radiation doses near their LD₅₀ levels die from bacteremia (Lorenz and Congdon, 1954; Miller et al., 1951)). Based on this analysis, it is hypothesized that the propensity to develop DIC has a role in determining the LD₅₀

for the mammalian species. This hypothesis provides a novel and reasonable explanation for the great variability observed in LD₅₀ values among different mammalian species.

There is a considerable amount of evidence that humans exhibit hemorrhaging at doses near the LD₅₀. The LD₅₀ of the atom bomb casualties has been estimated to be approximately 2.5 Gy (Lushbaugh, 1969; Fujita et al., 1991). The percent of those dying from exposure to the atom bombs with evidence of hemorrhage can be estimated from the atom bomb casualty data, which has been reviewed by Liebow et al. (1949). The casualty dose estimates are given in a report by the U.S. Atomic Energy Commission (USAEC, 1951). As described by Liebow et al. (1949), people exposed to radiation from the atom bomb used in Hiroshima have been classified into several groups. In Group II (patients dying during the third, fourth, fifth and sixth weeks or surviving with severe clinical symptoms), some of the people lived and some of the people died. It is expected that the people who died in Group II were those who received a dose of radiation near the human LD₅₀ level. The data that were collected from each person represented one tissue section from each tissue/organ examined, and the fraction of tissues exhibiting hemorrhage is reported by Liebow et al. (1949). For many of the organs/tissues (e.g., kidney, liver and other organs), the percentages exhibiting hemorrhage are as high as 60%, but it is not clear what fraction of exposed individuals experienced hemorrhaging in one or more organs. (For example, it is not indicated whether 60% of the livers exhibiting hemorrhage are from the same people as 60% of the kidneys exhibiting hemorrhage). Therefore, it is assumed that 60% of the people exhibiting hemorrhage is a low estimate and that the true value lies between 60% and 100% of the people dying in Hiroshima (after exposure at a dose near the LD₅₀) that had evidence of hemorrhage. These data indicate that the atom bomb casualties are likely to have had very high frequencies of hemorrhaging at death, similar to those observed for other large mammals (e.g., pigs and dogs).

It is also noteworthy that knowledge obtained about the adverse health effects of inhomogeneous doses of interventional and therapeutic radiation is relevant for evaluation of radiation effects in people resulting from exposure to interventional radiology procedures or therapeutic radiation, in which the skin doses are higher than the internal organ doses. An example of the relevance for patients on earth is that high doses of SPE-like radiation primarily to the skin with minimal to no significant doses to internal organs have been shown to produce adverse health effects in internal organs (e.g., pulmonary toxicities or pneumonitis with coughing in the lungs, bone marrow changes and reductions in circulating blood cell counts (Wilson et al., 2011)).

7. Summary concerning the major effects of SPE and space radiation

7.1. Dosimetry

With the incorporation of modern radiation oncology approaches, such as CT based Monte Carlo dosimetry, into the Human Space Program, it has become feasible to accurately predict organ specific radiation doses for astronauts exposed to SPE radiation. Depending on the organ system of interest (deep vs. superficial) and the fluence/energy profile of the exposure (hard vs. soft event), either the physical size of the astronaut or the fluence/energy profile for the SPE can be the determining factor for radiation induced dose/toxicity.

7.2. Adverse effects in hematopoietic/immune system cells

Significant decreases in WBC counts were observed in mice and ferrets irradiated with SPE-like proton and γ -ray radiation at total body doses of 0.5 to 2 Gy, and in pigs irradiated with proton

simulated SPE radiation at skin doses of 2 to 10 Gy and electron simulated SPE radiation at skin doses of 2.5 to 25 Gy. At the higher doses of proton or γ -ray radiation, the neutrophil counts in the blood of both mice and ferrets, but not pigs, reached critically low levels that, if observed in a patient in a hospital (e.g., following radiation or chemotherapy for cancer), would trigger a medical response and suggest the use of countermeasures to increase the level of neutrophils. In the pigs exposed to proton simulated SPE radiation, the neutrophil counts did not return to normal levels even at months after the radiation exposure.

7.3. Adverse effects on the immune system

At a dose of 2 Gy, SPE-like proton radiation or γ -ray radiation was shown to cause breaks in the epithelial layer of the small intestine of mice, which allows translocation of bacteria and bacterial products, such as LPS. The threshold for the morbidity/mortality effect in bacterial challenge studies was between 1.0 and 1.5 Gy. The proton radiation works synergistically with HS to result in an accumulation of a relatively large amount of LPS in the intervillous regions of the ileum. Due to the synergistic effect from HS, the threshold doses for immune system parameters determined without the HS treatment may underestimate the actual immune system risks for astronauts during space travel involving microgravity conditions.

Another major adverse effect observed in immune system cells is the lack of, or reduction in the level of, activation in T-lymphocytes in mice exposed to 1–2 Gy of SPE-like proton or γ -ray radiation, either with or without simulated microgravity (using the PWS and HS systems).

7.4. Emesis (vomiting and retching)

In female descended Fitch ferrets, irradiation with ^{60}Co γ -rays or 155-MeV protons at a high dose rate of 0.5 Gy/minute resulted in dose-dependent changes in the endpoints that are indicative of retching and vomiting. The minimum radiation doses required to induce statistically significant changes in retching- and vomiting-related endpoints were 0.75 and 1.0 Gy, respectively. The RBE of the proton radiation at the high dose rate (relative to the γ -rays at the same dose rate) did not differ significantly from 1. Similar, but smaller and less consistent, changes in the retching- and vomiting-related endpoints were also observed for ferrets irradiated with γ -rays and protons at the low dose rate of 0.5 Gy/hour. Since this low dose rate is similar to a radiation dose rate expected during a SPE, these results suggest that the risk of SPE radiation-induced vomiting is low and is likely to reach statistical significance only when the radiation dose reaches 1 Gy or higher.

7.5. Skin effects

At skin doses of 7.5 Gy and higher, there is a persistent immunological dysfunction in pig skin, which is characterized by an enhanced DTH response; a similar effect was observed in mice at doses of 2 Gy or less. At higher doses of radiation, the pig skin becomes more sensitive to touch (lymphedematous), and there can be blistering, burns and epithelial dysfunction (e.g., pigment incontinence, which indicates defective cell to cell transfer of biomolecules). The threshold radiation doses for the skin effects are 4.5 to 5 Gy. The decrease in vascular bed area is the most serious skin effect potentially resulting from irradiation at very high skin doses. However, this complication would be a highly unlikely occurrence for astronauts since such high radiation doses are not expected during most SPEs.

7.6. Disseminated intravascular coagulation

Both SPE-like proton and γ -ray radiation at doses near 2 Gy resulted in DIC in ferrets, which led to 100% mortality. The threshold dose of radiation for this effect is 1.5 Gy. This is an important finding, as DIC has not been established previously as a cause of death in mammals following radiation exposure. The blood clotting abnormalities in ferrets were observed at a very low dose (0.25 Gy, which was the lowest dose evaluated in the studies), and radiation at the SPE-like low dose rate resulted in more severe effects on the blood clotting parameters in ferrets than the high dose-rate irradiation.

Yucatan minipigs exposed to a 2.5 Gy dose of radiation were also diagnosed with DIC and died (or were euthanized). Minipigs exposed to a 2 Gy dose of SPE like proton radiation did not die, but they exhibited severe clotting abnormalities (increased bleeding times) that could be problematic during space travel.

Based on the work in ferrets and pigs performed as part of the space radiation studies described here, it is hypothesized that DIC may be a major cause of death in humans following exposure to relatively low doses of radiation.

7.7. Changes potentially related to the development of vision abnormalities

It was observed that pigs exposed to 2.5–7.5 Gy (skin dose) of simulated SPE – electron radiation (October 1972 event) exhibited increased opening pressure values, which lasted up to 90 days post-radiation. Other endpoints have also been evaluated which could have significance for the findings that many astronauts exposed to long-duration space flight have exhibited vision alterations. As one example, increased nerve sheath diameters (documented by ocular ultrasound technology) have been observed in pigs exposed to SPE-like radiation. Increased nerve sheath diameters have also been documented by ocular ultrasound technology in astronauts and this finding has been implicated in the development of astronaut vision alterations (Mader et al., 2011).

A relatively low dose of SPE-like radiation (skin doses as low as 2.5 Gy) can lead to increased intracranial pressure in pigs, and a dose of 5 Gy of proton simulated SPE radiation was shown to increase the nerve sheath diameter. The statistically significant effects observed on these endpoints in the pig studies suggest that astronaut exposure to these relatively low doses of SPE radiation could exacerbate the vision alterations known to exist in astronauts during future exploration class missions.

7.8. Threshold radiation doses for statistically significant adverse biological effects from SPE-like radiation in vivo

The estimated threshold radiation doses to cause significant adverse biological effects *in vivo* for various endpoints measured are summarized as follows:

- Reductions in WBC counts in mice and ferrets: a homogeneous dose of 0.5 Gy; in pigs: a homogeneous dose of 2.0 Gy (lowest dose evaluated) of SPE-like proton radiation.
- Levels of death in mice exposed to SPE like protons, along with HS, in response to a bacterial challenge: 1.0 to 1.5 Gy.
- Elevated levels of LPS and LPB in mouse serum: 2 Gy of SPE-like proton radiation.
- Increases in blood clotting time in ferrets: 0.25 Gy of SPE-like protons at a low, SPE-like dose rate.
- Lack of T cell activation in mice: 1 Gy.
- Skin effects: skin hyperpigmentation, epidermal thickening, and decrease in vascular bed area: 4.5 to 5 Gy of pSPE or eSPE radiation.

- Emesis in ferrets (for high dose-rate irradiation): 1 and 0.75 Gy for vomiting and retching, respectively. For low SPE like dose-rates, the results were not statistically significant at doses up to 2 Gy for SPE like proton radiation compared to controls.
- Levels of death from, or signs of, DIC in ferrets or pigs. All ferrets died with signs of DIC at a dose of 2 Gy from SPE-like protons or γ -ray radiation. The threshold dose for this effect is 1.5 Gy. Ferrets are thought to be more susceptible to radiation induced DIC than humans, while the sensitivity of pigs to radiation induced DIC may be more like that of humans. In an experiment in which 3 Yucatan minipigs were exposed to X-rays at a dose of 2.5 Gy, one pig died and another pig was euthanized; both of these pigs had evidence of DIC. 2.5 Gy is a potentially lethal dose of radiation for humans as well. At an SPE-like radiation dose of 2.0 Gy, pigs had severe clotting abnormalities suggestive of DIC. With the other space stressors (e.g., microgravity, elevated levels of oxygen during EVAs and carbon dioxide in the spacecraft, etc.), the lethal human dose could be < 2.5 Gy and astronauts may be susceptible to the onset and progression of DIC at doses that could be received from exposure to SPE radiation. In any case, astronauts are likely to have bleeding abnormalities from very low SPE radiation doses (25 cGy or below), and they should avoid exposure to anything that could damage the skin integrity due to the bleeding risk following a sizeable exposure to SPE radiation.

7.9. RBE values

A higher RBE value for a given effect indicates that a more severe effect is expected for exposure to SPE radiation than from conventional reference radiations (e.g., γ -rays, x-rays or electrons). In our studies, it was observed that there can be: 1) different RBEs for different biological endpoints in the same animal species/strain, and 2) different RBEs for the same biological endpoint in different species/strains. However, for most of the endpoints evaluated, which include hematopoietic blood cell counts, immune system parameters and fatigue measured in mice, emesis evaluated in ferrets, skin effects assessed in pigs, the RBE values were not significantly different from 1 except for the following:

- Hematopoietic blood cell counts in ferrets: RBE ranges from 1.2 to 1.6 for the WBC count at 48 hours after irradiation, RBE ranges from 1.9 to 2.1 for neutrophil count at 48 hours after irradiation (RBE values were particularly increased at the low end of the SPE proton dose range evaluated);
- Hematopoietic blood cell counts in pigs: RBE ranges from 2.4 to 4.1 for the WBC count, and 2.2 to 5.0 for the neutrophil count on day-4 post-irradiation (RBE values were particularly increased at the low end of the SPE proton dose range evaluated).

RBE values were determined for hematopoietic cell counts in mice, ferrets and pigs; therefore, they can be compared across these three species. From these data, it was observed that the RBE values of the SPE-like radiation for white blood cell counts vary greatly between mice, ferrets and pigs, with the RBE values being somewhat greater in ferrets than in mice, and considerably greater in pigs than in ferrets or mice. This trend suggests that the RBE values of SPE radiation for white blood cell counts could be considerably greater in humans than those observed in smaller mammals, and that SPE proton radiation may be far more hazardous to humans than previously estimated from studies performed in small animals (e.g., rodents).

7.10. Dose-rate effects

One major endpoint evaluated that was affected by dose rate was ferret emesis, which was significantly increased after irradiation with SPE-like proton radiation or γ -ray radiation administered at the high dose rate of 0.5 Gy/minute, but not at a low dose rate of 0.5 Gy/hour. The other major endpoint affected by dose-rate was the blood clotting time, which was increased to a considerably greater extent in the studies with the low, SPE-like dose-rate than in the high dose-rate radiation studies. For all other endpoints, the sparing effects of the radiation dose rate are either insignificant or minimal and not biologically meaningful.

8. Agents identified as countermeasures for space radiation induced adverse biological effects

As a part of the work for this program, the following agents have been identified as countermeasures, which may lead to risk reductions in astronauts exposed to space radiations.

A. Dietary antioxidants (Wambi et al., 2008, 2009), fructose (Romero-Weaver et al., 2014) and G-CSFs (Neupogen, Neulasta) as countermeasures to prevent or alleviate the loss of circulating white blood cells (Wambi et al., 2008, 2009; Romero-Weaver et al., 2014), neutrophils (Wambi et al., 2008, 2009; Romero-Weaver et al., 2013a), and lymphocytes (Romero-Weaver et al., 2014).

B. Orally administered antibiotics (e.g., enrofloxacin) as a countermeasure to prevent or alleviate translocation of bacteria and bacterial products as well as death from bacterial challenge in animals exposed to SPE-like proton radiation and hindlimb suspension (Drew Weissman, Unpublished data).

C. 5-HT3 antagonists (e.g., oral ondansetron [Zofran]) as a countermeasure for proton radiation induced emesis (retching and vomiting) (King et al., 1999).

D. Corticosteroid therapy as a countermeasure for pneumonopathy/pneumonitis that developed after radiation exposure (Wilson et al., 2011).

E. Topically applied steroid cream (mometasone-Elecon) (Cengel, K.A. and Sanzari, J.K., Unpublished data) and transparent film dressing (Whaley et al., 2013) as countermeasures for radiation induced skin damage (e.g. hyperpigmentation).

F. Dietary antioxidants as a countermeasure to decrease the risk of long-term radiation effects, e.g. cancer (Kennedy et al., 2008, 2011) and cataracts (Davis et al., 2010).

G. Benefix as a countermeasure for increased bleeding times after radiation exposure (Krigsfeld et al., 2013).

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