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THE INDIVIDUAL AND COMBINED EFFECTS OF SPACEFLIGHT RADIATION AND MICROGRAVITY ON BIOLOGIC SYSTEMS AND FUNCTIONAL OUTCOMES

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Abstract

Both microgravity and radiation exposure in the spaceflight environment have been identified as hazards to astronaut health and performance. Substantial study has been focused on understanding the biology and risks associated with prolonged exposure to microgravity, and the hazards presented by radiation from galactic cosmic rays (GCR) and solar particle events (SPEs) outside of low earth orbit (LEO). To date, the majority of the ground-based analogues (e.g., rodent or cell culture studies) that investigate the biology of and risks associated with spaceflight hazards will focus on an individual hazard in isolation. However, astronauts will face these challenges simultaneously Combined hazard studies are necessary for understanding the risks astronauts face as they travel outside of LEO, and are also critical for countermeasure development. The focus of this review is to describe biologic and functional outcomes from ground-based analogue models for microgravity and radiation, specifically highlighting the combined effects of radiation and reduced weight-bearing from rodent ground-based tail suspension via hind limb unloading (HLU) and partial weight-bearing (PWB) models, although in vitro and spaceflight results are discussed as appropriate. The review focuses on the skeletal, ocular, central nervous system (CNS), cardiovascular, and stem cells responses.

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Keywords

Hind limb unloading; tail suspension; radiation; spaceflight; cognition; cardiovascular; SANS; bone; stem cell

1. INTRODUCTION

Both microgravity and radiation exposure in the spaceflight environment have been identified as challenges to astronaut health and performance[1–6]. While substantial study has been focused on understanding the biology and risks associated with prolonged exposure to microgravity and the hazards presented by radiation within and outside of low earth orbit (LEO), other hazards exist that can impair individual and team performance and well-being. These include the experience of isolation from social groups and networks in confined and extreme environments[7–10] combined with lack of sleep quality and quantity with disrupted circadian cycles[3,8,11–13]. NASA's research portfolio which examines spaceflight hazards to astronaut health and performance heavily rely on ground-based analogues using rodent models, with most studies focusing on a single hazard. There are ethical and obvious health limitations in performing controlled experiments that examine radiation and/or microgravity effects in astronauts. Hence, the use of ground-based analogues to study spaceflight hazards have been useful in gaining insight into both mission-critical and post-mission effects of spaceflight environmental challenges on astronauts' tissues, organs, systems, and functional performance. Astronaut health must be maintained in order to ensure success and quality of life as NASA directs resources to planned missions to the moon, Mars, and extended use of the International Space Station (ISS). The ultimate focus of this review is to describe physiologic outcomes from ground-based analogue models for microgravity and radiation, specifically highlighting the combined effects of radiation and reduced weight-bearing from rodent ground-based tail suspension via hind limb unloading (HLU) and partial weight-bearing (PWB) models, although *in vitro* and spaceflight results are discussed as appropriate and in comparison with HLU results. In addition, the limitations of the HLU model as a microgravity analogue, within and across systems will be discussed.

Loss of gravitational loading on the body and across tissues results in multisystem injury[6]. Among musculoskeletal tissues that exhibit a dynamic response to loading[14–16], exposure to microgravity results in: muscle atrophy[17,18], loss of bone mineral density primarily at load-bearing skeletal elements (e.g., pelvis, femur, vertebrae)[19–21], and increased risk of herniation of the intervertebral disks due to increased fluid volume[22,23]. These negative effects also could be compounded by spinal muscle atrophy[18] and affected spinal ligaments, ultimately leading to pain[23–25]. Cardiovascular responses to microgravity include redistribution of blood cranially, increasing arterial pressure at the head and lowering pressure at the distal leg elements[26]; though the response may be transient. This altered pressure gradient may contribute to spaceflight-associated neuro-ocular syndrome[3] in which optic disk edema, optic globe flattening, thickening or folding of the choroid layer, ischemic regions of the retina, and hyperopic visual disturbance may occur[27]. With these and other systems degraded with exposure to microgravity, as reviewed herein, ground-based

analogues are essential for identifying and developing countermeasures. To this end, the rodent HLU model is a primary surrogate for “microgravity”.

The HLU model was originally created as a ground-based analogue for reduced weight-bearing on skeletal tissues in order to study musculoskeletal deficits caused by spaceflight[28]. Briefly, the hind limbs are lifted off of the cage floor as the tail is tethered to a support bar within the cage[1,28,29]. Many studies use traction tape to tether and lift the tail and hind limbs off the ground; however other methods for tail suspension exist, for instance pinning of the tail to a tether[30]. The standard is to raise the tail so that the torso is at a 30° angle relative to the substrate to permit relatively normal forelimb loading, while decreasing load applied across the hind limbs[28]. Bone and muscle atrophy unsurprisingly occur with the reduction in weight-bearing[31–35]. This approach of tail suspension has been considered standard in part because it results in other physiologic responses analogous to the body’s response in microgravity, such as a cephalic fluid shift[28,36–38]. Because of the ensuing fluid shift, reductions in weight-bearing and resulting cardiovascular and metabolic adaptations within and across systems[2,39], this approach has been widely adopted as a microgravity analogue compared to other disuse models of osteoporosis, such as neurectomy/nerve crush to induce degrees of paralysis[40–42], or limb immobilization models[43,44].

While the HLU model remains the gold-standard for ground-based microgravity research, more recently developed models exist that permit investigating gravity as a continuum, using PWB via quadrupedal unloading in both mice[45] and rats[46–49]. The PWB model was principally designed to measure the dose-response relationship between degree of mechanical loading/weight-bearing and the musculoskeletal response and, thus far, studies have demonstrated a clear dose-dependent response but failed to detect the existence of a safe gravity threshold to minimize musculoskeletal deconditioning[50–52]. The rat PWB model uses of a pelvic harness in lieu of a traditional tail suspension, with limited obstruction of blood flow or creating a cephalic fluid shift as observed from existing studies[53], and represents an environment to assess new countermeasures, both therapeutic[54] and diagnostic[55]

Space Radiation Environment and Dose Estimates:

Space explorers outside of low-Earth Orbit (LEO) will be exposed to separate sources of ionizing radiation, energetic protons associated with a Solar Particle Event (SPE) and Galactic Cosmic Rays (GCR)[56,57]. NASA is currently planning for a sustainable presence on the lunar surface and exploration class missions to Mars, far away from the protection of Earth’s magnetic field. The nature of the radiation exposures that astronauts encounter will likely change to include higher GCR and possible SPE exposures[57].

GCR nuclei originate outside our solar system and are high-LET (linear energy transfer), ions with enough energy to easily pass through typical spacecraft with minimal energy loss[58]. The considerable ionization power of GCR ions makes them the primary antagonist for possible late effects to multiple physiologic systems. During spaceflight in interplanetary space, every cell nucleus will be traversed by a hydrogen ion or delta ray every few days, and by the heavier GCR ion every few months[59,60]. Despite their relatively low frequency, the heavy ions contribute a significant amount to the GCR dose that astronauts will incur

outside of LEO. Thicker shielding could provide protection but is limited by the capabilities of spacecraft launch systems. In fact, studies have shown that even if the amount of, e.g., aluminum, shielding is increased, there will not be a significant reduction of the intra-vehicular radiation dose[57,60,61]. Astronauts currently on the International Space Station (ISS) are exposed to on average about 1 mSv/day, and even have emergency plans to shield themselves with water bags should an SPE occur. As we travel further outside of LEO, however, it is expected that this daily dose will increase by a factor of 2–3 with limited additional protective measures for SPEs due to spacecraft design and consumables[57,62,63].

While background GCR radiation is a concern, SPEs represent an immediate acute exposure of radiation dosage especially if they occur during extravehicular activity. SPEs can produce energetic particles at levels that are orders of magnitude higher than ambient exposures[64,65]. SPEs consist of high-energy protons that emanate from the Sun from regions of magnetic instability[57,62]. The ability to predict the occurrence or magnitude of future SPEs, and the likely doses received by exposed crew, are limited[66,67]. The acute radiobiological effects of whole-body SPE exposures are not well understood. They are complicated by the inhomogeneous distribution of radiation doses to sensitive organs and difficulty with extrapolating animal model data to humans[56,57]. Additionally, it is unknown how the human health response to SPEs will be affected by concurrent GCR exposure[56,57]. Based on calculated exposures in the context above, even large SPEs are predicted to deliver doses of <0.5Gy-Eq to internal organs and skin doses of <2.5Gy, with rates of delivery peaking at a dose rate of approximately 0.12Gy-Eq/hr to blood-forming organs[66,68]. For an SPE similar in magnitude to the 1972 event, an astronaut crew would incur an intra-vehicular skin dose roughly equal to NASA's 1-year spaceflight radiation permissible exposure limit (3.0Gy-Eq/skin and 500mGy-Eq to blood forming organs[64,69,70]. Additionally, the dose to blood-forming organs would approach the 1-year permissible exposure limit for these organs (0.5Gy-Eq)[69].

Combined Radiation and Reduced Weight-Bearing Overview:

As astronauts will face multiple hazards simultaneously during long duration missions outside of LEO, it is necessary to test the individual and combined effects of these hazards in order to best determine health risk and develop countermeasures. To date, several ground-based rodent studies have combined HLU with radiation exposure to assess combined biologic effects[71–73]. As detailed herein, while the reduced weight-bearing model is generally HLU (with some PWB), radiation sources vary greatly (primarily photon exposures, with some proton and a few high-Z high-energy ions (HZE) exposures at NASA Space Radiation laboratory (NSRL)). Of note, the majority of studies have performed a period of HLU followed by radiation exposure or initiate the study with radiation exposure and then enroll rodents into HLU[35,71–78]. A few studies examine outcomes after simultaneously delivering radiation with HLU[29,31,72,73,79–84]. For these, rodents are irradiated in the beamline at the NSRL, or at other facilities with low-dose rate gamma rays or photons.

2. SKELETAL RESPONSE

Bone Loss in Microgravity and Reduced Weight-Bearing:

Spaceflight environmental conditions, particularly microgravity and low-dose radiation, represent a risk to astronaut bone health. In the absence of mitigation strategies, spaceflight can lead to decrements in bone mass and strength[85]. Crew members returning from a 4–6 month mission on the ISS had reduced areal bone mineral density (aBMD) of the hip and spine. The rate of bone loss was found to be site-specific, with a 1.4–1.5% and 0.9% decrease in aBMD of the hip and spine respectively per month in the ISS. In the hip, the rate of loss was higher in cancellous bone (2.2–2.7%/month) compared to cortical bone (0.4–0.5%/month)[86]. In contrast, trabecular vBMD and mass decreased by 14.4–16.5 % throughout the mission duration[87]. Bone strength in the proximal femur also was reduced as determined by finite element modeling for stance and fall loading[21]. These decrements in skeletal structure and strength may increase the risk of fractures, which in turn can be catastrophic to future long duration spaceflight missions where there will be operational constraints on medical care and equipment.

Animal models have been used to gain insight on spaceflight-induced bone loss and its underlying mechanisms. As noted, because of the dynamic response of bone to loading, one of the advantages of using animal models, particularly from ground-based HLU, is that brisk bone loss can be achieved (within weeks to months) upon exposure to simulated microgravity[28,36,39,88] and partial weight-bearing[45,48,50,89]. The magnitude of bone loss achieved by HLU varies across studies which is unsurprising given differences in methodologies, duration and age at onset of HU, as well as sex and strain of animals used. Seven days of HLU in 17 week old male C57BL/6J mice led to ~15% decrease in % cancellous bone volume (BV/TV) in the proximal tibia[78]. In female C57BL/6J mice, one month of HLU led to a 74% reduction in % bone volume in the proximal tibia[35]. A 21 day HLU period in 12 week old male C57BL/6 mice led to decreases in BMD as measured at different sites along the femur[90]. The magnitude of HLU-induced decreases in BMD ranged from 8–11% as measured from the proximal to distal end of the femur[90]. The appendicular bones of humans and rodents have notable differences in anatomy and stance loading, in addition to dissimilarities in the timing and duration of postnatal skeletal growth. Hence, anticipating the magnitude of bone loss in humans from a rodent model for skeletal disuse is not straightforward. Nevertheless, the results from the HLU model are generally consistent with findings in humans that microgravity can accelerate bone loss.

Space Radiation-induced Bone Damage:

Bone loss also has been examined in animal models for space radiation exposure. Historically, bone was considered radiation resistant, and thus the dose threshold for damage was assumed to be high[91,92]. However, studies performed in the early portion of the 2000's identified late bone loss in mice after 2 Gy exposures to multiple qualities of radiation (e.g., protons, iron ions, carbon ions, and photons), with the consideration that 2 Gy represented an appropriate comparative dose between clinical fractions and spaceflight exposures[93]. Later studies typically made use of a lower reference dose of ionizing radiation (e.g. 1–2 Gy ^{137}Cs)[73,81,94], a single representative species of GCR[35,84,95,96] or sequential exposure to two

ion species[72,97]. These spaceflight relevant studies identified that while osteoblast activity is lower after exposure (which historically was assumed to be the mediator of late bone damage after high dose exposure), osteoclast activity was increased as an early response[98,99], leading to acute bone loss[78,98]. The dose threshold for bone loss after simulated spaceflight radiation was determined to be low, with mixed fragment doses <0.5 Sv (protons and helium ions) causing late bone (and muscle) damage[100], and individual doses of 0.5 Gy ^{56}Fe and protons causing acute bone loss in mice[101]. These investigations have thus confirmed that bone is a radiation sensitive tissue, with the outcome being loss of architecture and density. However, one study has reported that radiation can have positive effects on bone microarchitecture[74]. Skeletally mature (16 week old) female mice exposed to 0.5 Gy ^{56}Fe exhibited increased cancellous % bone volume (BV/TV), trabecular thickness (Tb.Th.) and trabecular number (Tb.N.) in the distal femur at 21 days post-irradiation. These results highlight that while radiation is generally considered disadvantageous, many questions remain regarding how low dose radiation could affect bone, including questions regarding bone material and mechanical properties that occur with alterations in architectural properties. These questions should be addressed in the context of complex, mixed beams of energetic particles to improve the model spaceflight-relevant radiation exposures. Recently, the NSRL has developed a GCR simulation involving a more complex combination of multiple ion species that approximates the anticipated exposure of crew based on current spacecraft design[102].

Impact of Combined Exposure of Space Radiation and Simulated Microgravity on Bone Structure and Function:

Published studies on the combined effects of microgravity and radiation on skeletal integrity differ in experiment designs, making comparison and validation of results across studies quite challenging. Despite the limited number of reports and heterogeneity in experiment designs, two major conclusions can be drawn from collective findings. Firstly, findings from animal models predict that bone loss due to microgravity and ionizing radiation exposure can be additive under certain conditions. Secondly, skeletal parameters may have differential sensitivities to combined spaceflight factors. In some studies that have used representative high linear energy transfer (LET) species in combination with HLU, additive effects were seen in a subset of skeletal structural parameters but not in biomechanical properties. For example, 16-week old, male C57BL/6J mice that underwent 14 days of HLU displayed decreased cancellous and cortical BV/TV in tibia versus normally loaded controls while similarly aged cohorts exposed to 50 cGy proton or 50 cGy proton + 10 cGy ^{16}O did not show such decrements. Yet when HLU was combined with 50 cGy proton + 10 cGy ^{16}O exposure, further decrements in BV/TV were observed. Biomechanical properties such as maximal force, measured by three-point bending, was negatively impacted by HLU but not by radiation. However, no further deficits were observed when HLU and radiation were combined[72]. These findings are generally consistent with another report which made use of similarly aged female mice[35] exposed to 100 cGy of protons in combination with four weeks of HLU. Combined microgravity and radiation also can lead to skeletal impairments at the cellular level. Skeletal homeostasis is achieved through the balance in the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Exposure to 0.5 Gy ^{56}Fe was found to exacerbate HLU-induced deficits in alkaline phosphatase activity of marrow-derived ex vivo

osteoblast cultures, suggesting that microgravity in combination with radiation exposure can impair osteoblastogenesis[96]. Additionally, two weeks of HLU combined with 1 Gy ^{56}Fe delivered on Day 3 led to enhanced bone loss versus exposure to each single hazard. This study also identified potential mechanisms for bone loss in the hind limb from each challenge, related to endothelial dependent vasodilation of the feed arteries of the gastrocnemius[83]. Specifically, it was identified that altered vasodilation associated with bone loss was mediated by altered nitric oxide (NO) synthase signaling, although occurring in divergent manners between hazards: with HLU, alterations occurred to reduced production and concentration of NO, and radiation-induced alterations were due to increased NO quenching.

Long duration missions will involve extended periods of exposure to low doses of space radiation. Most of the available data on the effects of radiation on the skeleton were generated from acute exposures. Published results from fractionated or sustained exposure models remain a rarity. One group has examined the effects of 0.5 Gy ^{28}Si delivered as an acute dose or three fractionated doses of 0.17 Gy in combination with PWB at one sixth body weight[95]. The PWB-induced decrements in both endocortical and periosteal % mineralizing surfaces of cortical bone sites appeared to be worsened by ^{28}Si although standard deviations were wide, therefore necessitating additional confirmatory studies[95]. In general, neither acute nor fractionated exposure exacerbated the effects of partial weight-bearing on femoral BV/TV and femoral neck biomechanical properties such as load to failure and stiffness. Others who have simultaneously applied continuous exposure to low dose rate photons (8.5cGy ^{137}Cs) exhibited no radiation response, only bone loss due to HLU over the course of a 20 day study[81].

The long-term consequences of combined microgravity and radiation on skeletal health is unclear given that most investigations have focused on immediate time points (e.g. assessment after unloading). However, there is evidence that combined exposure to HLU and HZE (0.5 Gy) can negatively impact skeletal recovery after a period of unloading. In one such study[84], animals that received an acute dose of 0.5 Gy ^{56}Fe within a 14-day period of HLU displayed persistent deficits in vertebral trabecular morphology (structural model index, SMI) after a 28-day period of re-ambulation. However, cancellous BV/TV of vertebra of these animals were comparable to untreated controls after the same time period.

Humans living in space experience circadian misalignment[103]. The interaction of the circadian clock with microgravity and space radiation has not been well examined with alterations in bone as an outcome. One investigation exposed rats to an acute, high dose of radiation (4 Gy ^{137}Cs), with ultradian cycles (45 min light/45 min dark), HLU or all factors combined. Ultradian cycles were sufficient to induce decrements in bone biomechanical properties but did not lead to bone loss. In addition, ultraradian rhythms did not worsen the negative effects of HLU and radiation on bone structure and strength.

Countermeasures in place to prevent osteopenia during ISS missions include load-bearing exercises. Strides also have been made in identifying other promising candidate countermeasures for mitigating spaceflight-induced bone loss. Antioxidants are often considered as candidate countermeasures to protect against radiation and/or HLU-induced

bone loss: as previously noted altered NO concentration (and expanded upon in Section 5; Cardiovascular Response) is associated with radiation and HLU[83], and biomarkers for oxidative stress in the marrow in another study has been identified to increase after radiation but not HLU[78], highlighting some inconsistencies in the literature. In a rodent model, pre-feeding with an antioxidant-rich dietary supplement (dried plum) prevented bone loss and decrements in bone strength resulting from HLU, ionizing radiation and in combination[94]. These results suggest some shared mechanisms underlying microgravity and radiation-induced bone loss. However, while antioxidants such as dihydrolipoic acid (DHLA) or an antioxidant cocktail have been shown to be less efficacious at reducing bone loss in mice early after 2 Gy gamma ray exposure,[97] alpha-lipoic acid[78] has been shown to reduce early bone loss after 2 Gy gamma rays. Collectively, these findings suggest that antioxidants have varying efficacies in preventing the negative effects of spaceflight stressors on bone. Future studies are needed to better understand the underlying mechanisms for the protective effects of promising antioxidant-based countermeasures for spaceflight.

Additional and Future Considerations:

Although much has been achieved in understanding the effects of spaceflight on bone health, follow-up studies are needed to validate the abovementioned findings using improved radiation exposure paradigms such as the recently developed multi-ion GCR simulation[102]. The use of animals that better match the age of mission crew when performing spaceflight simulation studies will facilitate translation of rodent data to humans in space. In addition, standardization of methodologies and caging designs for conducting HLU and partial weight-bearing studies also are important to allow for comparison and interpretation of findings across studies and research groups. More mechanistic investigations also are needed to understand the molecular underpinnings of the skeletal response to combined spaceflight factors. Bioinformatics approaches that allow for the assessment of global changes at the genome, transcriptome and proteome levels are particularly useful in identifying novel pathways that underlie the skeletal response to spaceflight. To date, omics data from skeletal tissue is rare. Of the two traditionally mechanosensitive tissues, muscle vastly outnumbers bone or bone cell transcriptomic datasets found on the NASA GeneLab database. Increasing the availability of bone omics datasets will facilitate improved understanding of skeletal signaling in response to spaceflight.

Initiatives to study bone health in space have predominantly focused on how spaceflight negatively impacts skeletal structure and biomechanical properties with the overarching goal of understanding how they contribute to fracture risk. While it is important to address remaining knowledge gaps on fracture risk in follow-up studies, investigations by the space research community also need to broaden to reflect our growing understanding of skeletal function. Beyond providing structural support, the skeleton is an endocrine organ that can crosstalk with other tissues to maintain health and homeostasis. A number of studies demonstrate the ability of bone to function as an endocrine organ (reviewed in[104–106] to regulate a variety of physiological processes including glucose metabolism[107–109], appetite suppression[110], cognition and behavior[111,112]. The organism's ability to coordinate the function of multiple tissues via bone-derived factors is essential to keep up with the demands of daily living. For example, bone-derived lipocalin-2 (LCN2) regulates glucose homeostasis

via endocrine action on major metabolic organs, and also can cross the blood-brain barrier to control appetite via its binding to the melanocortin receptor (MC4R) in the hypothalamus[110]. In addition, LCN2 can exert pro-inflammatory actions on a variety of cell types including vascular cells[113]. Osteocalcin (OCN), another bone-derived hormone can modulate cognition and anxiety-like behavior[111,112]. Mice heterozygous for an OCN null allele showed deficits in cognition while administration of OCN improved memory and decreased anxiety-like behaviors[112]. In addition, OCN has been shown to mediate aspects of the acute stress response. In the presence of stressors, it is thought that OCN participates in signaling to inhibit the parasympathetic branch of the autonomic nervous system to allow the sympathetic pathway to predominate, in turn promoting flight or fight responses[114]. The biological processes regulated by these bone-derived hormones are critical for human health and performance in space missions. Hence, it is important to begin to address whether combined spaceflight factors can perturb signaling mediated by bone-derived hormones. More studies also are needed to understand the role of bone crosstalk with other organs in mediating the physiological changes attributed to spaceflight.

3. OCULAR RESPONSE

There has been an increase in the incidence of the ocular problem reported in astronauts during and after space shuttle missions or orbits aboard ISS[3]. As previously noted, this syndrome, known as spaceflight associated neuro-ocular syndrome (SANS), is characterized by pathophysiology symptoms including optic disc edema, globe flattening, choroidal and retinal folds, hyperopic refractive error shifts, and nerve fiber layer infarcts (i.e., cotton wool spots)[115–117]. In the last decade, over 30% of astronauts flying long-duration ISS missions have presented with one or more of these ocular disturbances[118]. Most recently, the NASA twin study found variations in choroidal and total retinal thickness, which was suggestive of retinal edema and choroidal folds in the twin exposed to spaceflight[119]. There is concern that degradation of visual function as a result of space flight may compromise both mission goals and long-term quality of life after space travel.

Ocular damage and retinal degeneration can be promoted by many factors including aging, ischemia, fluctuation in oxygen tension, oxidative stress, and increased intraocular pressure[120]. Visual disturbances associated with space travel may be due to exposure from altered gravitation changes and ionizing radiation[116,120–122]. Although some ocular changes experienced by astronauts have been measured and evaluated[117,118], validation was difficult due to the limited subject cohort size and test constraints on ISS. Furthermore, the underlying mechanisms of these ocular disturbance and factors contributing to the development of damage are currently unclear. Comprehensive ground-based rodent study models to simulate space condition including low-dose ionizing radiation and microgravity is warranted to determine the impact of the space environment on ocular structure and function.

Microgravity-induced Ocular Damage:

Microgravity induces a cephalic shift in body fluids, an increase in cephalad shifting of body fluids, and alterations in tissue perfusion[121,123,124]. Increasingly, evidence suggests that both

actual microgravity encountered by astronauts in space, as well as modeled microgravity on Earth, have been shown to induce many deleterious physiological effects including changes in ocular structure and function[125,126]. After long-duration spaceflight, morphological changes in the optic nerve and surrounding tissues have been reported[127]. One study showed that even in transient microgravity conditions, as produced by parabolic flight, changes in retinal vasculature occur[117]. Microgravity may also induce an increase in intraocular pressure (IOP)[128]. A more recent study reports that in astronauts, there is an acute increase in IOP upon entering weightlessness, but that it normalizes to ground-based levels after a few days of flight[118]. Despite reported observations, cellular mechanisms of microgravity in inducing the unique physiological and pathological ocular responses have not yet been well-understood. Moreover, head-down tilt (HDT) during bed rest as a ground-based, human analog for microgravity has not confirmed some of SANS findings in astronauts[129].

Space Radiation-induced Ocular Damage:

The adverse effects of radiation on the retina[130–133] and retinal vasculature[132,134] have been reported by multiple investigators who have documented structural, histopathological, and functional alterations in the affected retina after irradiation exposure. Despite reported studies, most of the animal and clinical investigations from which the current knowledge of radiation-induced ocular injury were obtained, are from relatively high doses of photon radiation. More recently, some ground-based studies have been conducted in rodent models to investigate low-dose space radiation-induced changes of ocular structure and function. Studies show dose-dependent increases in apoptosis in the retina following space radiation exposure. Data revealed that exposure to proton radiation-induced oxidative stress and apoptosis in the retina at a dose as low as 0.5Gy[124]. Analysis of the microvasculature in the rat retina showed a time- and dose-dependent, progressive loss of endothelial cells and microvessel length over for two years after proton irradiation[135]. Low doses of ^{16}O ions also elicited apoptosis in the mouse retinal endothelial cells with the most robust changes observed after 0.1 Gy exposure compared to controls[136].

Impact of Combined Exposure of Space Radiation and Simulated Microgravity on Ocular Structure and Function:

One of the main concerns for long-term deep manned space missions are health risks associated with combined exposure to microgravity environment and low-dose/low-dose-rate (LDR) radiation above levels normally found on earth due to GCRs. It is an important contribution for risk assessment to determine whether the low dose radiation response is modulated by simulated microgravity. The study design using a ground-based animal model to assess the biological effects of the spaceflight condition, combining space-like radiation exposure and microgravity is a more accurate model to simulate environmental stressors inherent to the spaceflight environment, providing a more actual risk assessment for astronauts. To our knowledge, ground studies to examine the impact of the simulated space flight conditions and underlying mechanism(s) of potential interaction on retinal structure and functional damage are very limited.

In one study, mice were HLU for 7 days, then whole-body irradiated with protons at 0.5 Gy, followed by HLU for an additional 7 days. The data showed SPE-like exposures of proton

irradiation alone or combined with simulated microgravity has a significant impact on retinal endothelial cell survival[71].

Additional and Future Considerations:

As noted earlier, factors and their interactions that contribute to detrimental ophthalmic changes to the spaceflight environment are not well-investigated. More studies are needed to expose animals to microgravity simulation and low-dose space radiation simultaneously over at least 4 weeks which simulate the duration of the ISS mission. This will allow the data to be extrapolated more accurately to estimate potential risks to astronauts in the space flight environment. To simulate key aspects of space radiation exposures, further rodent study designs should consider exposing animals to both the charged particle composition of the radiation field and its low dose rate for ocular response measurements. The observation periods for the study especially for degenerative tissues need to be extended for a longer time after radiation/unloading exposure to characterize the readaptation and chronic deficits. One of the complications associated with determining the response of stress insults is the latency between exposure and the expression of injury (e.g., cell loss or dysfunction). In order to obtain accurate data for the development and progression of the injury response, it is necessary to quantify changes over a long period.

The most profound physiological response and adaptation to the microgravity environment is the redistribution of fluid[121]. The mechanisms by which fluid shift in the spacecraft environment that could affect ocular function were less studied. Dedicated studies are needed to identify models to address this important question regarding the impact of the fluid shift on ocular structure, physiology, and visual function. Further studies are also needed to specifically assess ocular perfusion pressure and ocular hemodynamics in appropriate animal models. Electrophysiological assessment using electroretinogram (ERG) or other functional endpoints will be helpful to determine retina functional changes related to observed structural alteration.

Underlying cellular mechanisms of spaceflight environment, in facilitating ocular damage remain unclear. Some lines of evidence suggest that one of the mechanisms involved in response to spaceflight, including changes in the gravity vector, is likely due to oxidative stress[137,138]. Studies have shown that exposure to microgravity during spaceflights is associated with increased oxidative stress markers reflecting damage in lipid which results in lipid peroxidation in both humans and rodents[139,140].

To expand our knowledge about the effects of spaceflight condition on the eyes and possible mechanisms associated with these changes, integrated omics profiling technologies such as genomics, proteomics and metabolomics are beneficial to determine sets of differentially expressed genes (DEGs), differentially expressed proteins, metabolomic/lipidomic signatures and the pathways that lead to pathological and possible degenerative changes. Recently, RNA sequencing from a spaceflight study detected 600 DEGs in murine spaceflight retinas, which were enriched for genes related to visual perception, the phototransduction pathway, and numerous retina and photoreceptor phenotype categories[141].

Delineating differential gene and protein expression and their relationship to overall pathophysiological and functional changes in the ocular tissue will provide a basis for the discovery and development of biomarkers and pathways for neurovascular changes in response to spaceflight condition. Herein, using the “omics”-based molecular phenotyping approach for characterizing biosignatures associated with low-dose space radiation, simulated microgravity, and other space environmental stressors will help a deeper understanding of the underlying mechanisms responsible for the ocular structural and pathophysiological changes.

4. CENTRAL NERVOUS SYSTEM (CNS) RESPONSE

Importance of CNS Functionality to Mission Success:

Any deterioration in the ability of the astronauts to perceive or respond to changes in their situation could have disastrous consequences, as could changes in the mental health of the astronauts. The ability of astronauts to successfully complete a deep space mission, such as the ones planned for Mars, will thus be highly dependent upon a fully functional CNS. Not surprisingly, NASA has devoted considerable efforts to establishing the impact of social isolation, stress, sleep disturbances/loss and microgravity on various aspects of astronaut performance (cognition, sensorimotor response, social interaction, sleep) both during space flight and in rodent ground-based analogs. In many cases there are well established procedures and interventions to detect and mitigate these stressor related issues. However, there remains a high degree of uncertainty about the impact that exposure to space radiation will have on the cognitive and psychological capabilities of astronauts.

Microgravity Effects on the CNS:

Microgravity is also a major stressor on the CNS, inducing changes in the structure of the brain (rotation of the cerebral aqueduct, changes in ventricular volume, and narrowing of cerebrospinal fluid (CSF) spaces at the vertex[142], and a cephalic fluid shift. It also produces significant effects on the brain, particularly in cerebellar, sensorimotor, and vestibular brain regions (Reviewed in[143]). Brain activity may also change in response to the need for increased processing required for postural stabilization, and integration of conflicting vestibular information in the microgravity environment[144]. Despite all these changes, the evidence that prolonged microgravity leads to a permanent loss of cognitive function is sparse. Astronauts report a “Space fog” for 1–2 days into a mission, but this typically resolves.

However, at the cellular level, simulated[145] microgravity result in persistent changes in the mitochondrial function and lipid metabolism of human oligodendrocytes. Oligodendrocytes are essential for providing metabolic support to neurons, rapidly transferring (through cytoplasmic “myelinic” channels and monocarboxylate transporters) short-carbon-chain energy metabolites like pyruvate and lactate to neurons[146]. Such microgravity induced metabolic perturbations are likely to be deleterious to neuronal function in their own right, but will likely exacerbate changes in neuronal functionality with combined exposure to other spaceflight environmental hazards, such as radiation.

Space Radiation alters Neurophysiology and Neurocognitive Performance:

There is an ever-growing body of evidence from ground-based rodent studies that radiation exposure impairs performance in many cognitive processes, ranging from relatively fundamental processes to complex analogs/homologs of human cognitive tasks. Even in the one study that demonstrates an apparent radiation-induced improvement in pattern separations skills[147], mice demonstrated reduced associative memory formation ability, and the apparent improvement may be attributable to an increase in sparsely encoded hippocampal-dependent memory. Thus, the overall consensus from ground-based rodent studies is that radiation exposure impacts performance in multiple cognitive tasks, utilizing multiple cognitive process governed by multiple brain regions. Mechanistic studies have revealed multiple changes in neurophysiological processes and dendritic structure within most brain regions investigated[148–159]. Moreover, there may be a loss of connectivity between brain regions[149].

Space Radiation Alone Impacts Cognitive Processes Deemed to Be of Operational Significance:

While it could be argued that the loss of the ability to perform a task like novel object recognition may not have any operational significance, the radiation-induced loss of performance in rodent versions of tests widely used to assess attention and cognitive flexibility in humans cannot be so easily discounted. Astronauts are routinely screened during space flight for performance in a 10-test battery of cognitive tasks that NASA deemed necessary for mission success. Seven of the tasks in the “fit-for-duty” performance battery[160,161] assess some aspect of executive function. Executive function, in lay terms, can be summarized as the “Triple A”: the ability to Assess, Adapt and Achieve. More technically, executive functions are a set of higher order cognitive abilities that animals utilize to keep information ‘in mind’, attend to appropriate cues (e.g., nonverbal and verbal working memory stimuli), update information as contingencies change and invoke alternative, more appropriate responses to new situations.

The rodent version of the psychomotor vigilance test (rPVT) is virtually identical to the PVT test that is part of NASA’s “fit-for-duty” performance battery on the ISS[160,161]. PVT performance is sensitive to fatigue, drug use, and age[162,163], and exposure to mission-relevant (25 cGy) doses of protons results in deficits in accuracy, impulsivity and lapses in attention, all of which are indicative of deficits in sustained attention[164]. Such lapses in attention account for 80% of flight accidents in the Navy and Marine Corps[165].

A key process that allows humans to rapidly and efficiently adapt to different situations is task- or set-shifting. An attentional set is formed when complex stimuli must be discriminated and classified as relevant or irrelevant to a particular task/situation. Set-shifting can be simplistically thought of as the ability to relearn what the most important discriminating stimulus (for a particular endpoint) is in a changing environment. Attentional set shifting (ATSET) thus enables subjects to rapidly adapt and respond to changes in the environment, and to perceive what is important for survival or completion of a task, skills that are required to deal with a sudden emergency. The Wisconsin Card Sorting Test (WCST) has been widely used to assess task switching in humans[166], with task switching

deficits being increased in patients with Parkinson's disease[167,168] and autism[169]. The intra-dimensional (IDS)/extra-dimensional (EDS) set shifting task is a modification of WSCT that assesses set-shifting abilities in rodents[170]. Performance in the ATSET assay is impaired after exposure to ≤ 15 cGy of 1 GeV/n ^{56}Fe[155,171], 1 GeV/n ^{48}Ti[150,151], 600 MeV/n ^{28}Si[148] and protracted low dose rate neutrons[172].

Astronauts on deep space missions will have to act more autonomously than on previous missions, especially when rapid responses to unexpected problems are required. Creative problem solving skills will thus be of great importance to astronauts on a mission to Mars. Recent studies have shown that low (≤18 cGy) doses of 600 MeV/n ^{28}Si and ^{252}Cf-generated neutrons impact creative problem solving in rats[148,172]. Rather worryingly at the individual level, poor creative problem solving performance in the irradiated rats was not necessarily associated with poor ATSET performance, and vice versa[148,172]. Previously we have shown that while space radiation exposure impairs both spatial memory and ATSET performance, however, when the relative performance of individual rats in each task was compared there was no correlation between space radiation-induced loss of performance in each task[173]. These data suggest that risk assessments for radiation-induced neurocognitive impairment derived from a single cognitive domain may greatly underestimate the severity of the problem.

Establishing Space Radiation-resilience and Incidence of Severe Cognitive Impairment:

A notable feature of the rat-based studies was the marked inter-individual differences in cognitive flexibility performance of the irradiated rats, with some rats having performance metrics comparable to shams, while others completely fail to reach criterion in the cognitive tasks[148,151,155,164,171]. Analysis of the individual animal performance data using kernel density estimation to generate a performance probability profile, has allowed for an estimation of how frequently severe cognitive impairments are induced[171,172,174]. In these studies, the level of performance that was considered to represent severe ATSET impairment was set at the 5th percentile of the sham cohort performance profile (conceptually analogous to a Z-score of −2). Translating ground-based rodent studies into tangible risk estimates for astronauts remains an enormous challenge, but should similar neurocognitive impairments occur in astronauts exposed to low space radiation doses, a Numbers-Needed-to-Harm analysis (of the rodent data) predicts that ~30% of the astronauts could develop severe cognitive flexibility decrements[171,172].

Combining Spaceflight Hazards on Space Radiation-induced Cognitive Impairment:

To date, this is a largely uninvestigated, but much needed, field of study for the effect of simulating multiple aspects of the space environment on behavioral impairment. Relatively little has been documented regarding cognitive functioning after extended exposure to combined spaceflight condition, and findings have been inconsistent. One study showed that mice exposed to HLU displayed behaviors suggesting abnormal exploration and/or high risk-taking behavior in the elevated zero maze. However, low-dose radiation exposure did not exacerbate HLU-induced behavioral changes where mouse was exposed to combination of low-dose gamma radiation and simulated microgravity by HLU[175]. In an *in vitro* study, synergistic changes of reduction in neuronal network integrity and cell survival induced by

simulated space radiation and microgravity were observed[176]. Another study documented that learning and memory abilities were significantly reduced in rats under a simulated spaceflight hazards including microgravity, isolation confinement, noises, and altered circadian rhythms[177]. The potential interactions between space radiation, microgravity and other space environmental stressors should be addressed with comprehensive models including neuroimaging, electrophysiology, biological, and clinical data[178].

Additional and Future Considerations:

Both sleep deprivation and sleep fragmentation (SF) have been linked to reduced neurocognitive functioning in humans and animals, and has a major impact on performance in multiple cognitive domains[179–182]. Two recent studies have established how inadequate/disturbed sleep alters the severity of space radiation-induced impairment of executive function. A single session of fragmented sleep uncovered latent ATSET performance deficits in rats exposed to both protracted neutron[172] and Si[183] irradiation that had no obvious defects in performance under rested wakefulness conditions. SF selectively impaired performance in the more complex set shifting stages of the ATSET test in the both neutron and Si irradiated rats. Set shifting performance has rarely been impacted by exposure in studies conducted with rats tested under rested wakefulness conditions. Thus, radiation-induced cognitive impairment may not be fully evident in normally rested rats, substantially underestimating the level of impairment that may occur when astronauts are on mission. Cognitive testing may thus have to be conducted under both rested wakefulness and SF conditions to get a more accurate assessment of radiation-induced neurocognitive impairment. Further ground-based studies on the impact of radiation on cognition in rodents that are under concomitant exposure to space flight stressors such as stress and social isolation are being initiated. Based upon the exacerbating effects of sleep reduction on the incidence, severity and type of cognitive impairments induced, it would seem likely that concomitant exposure to these other space flight stressors would result in similar “negative interactions”.

5. CARDIOVASCULAR RESPONSE

Cardiovascular Response in Microgravity and Reduced Weight-Bearing:

The vascular endothelium is an important regulator of vascular tone in arterial, venous, and lymphatic vessels, and is vital for the protection of arteries from the development of atherosclerotic plaque. The production of NO by the vascular endothelium through the NO synthase (eNOS) signaling pathway is a critical component for these vascular functions. Limited information is available regarding the effects of spaceflight on the vascular endothelium in humans. Lee et al.([184]) reported that long-duration spaceflight in low Earth orbit elevated biomarkers of systemic oxidative stress and inflammation during flight, and these circulating biomarkers returned to preflight levels soon after landing. Despite the increase in systemic oxidative stress and inflammation, the astronauts showed no corresponding change in brachial artery endothelium-dependent vasodilation. Although these results suggest that spaceflight-induced elevations in oxidative stress and inflammation do not adversely impact vascular endothelial function, it is important to note that the source of the circulating biomarkers is unknown and may originate from specific or unique portions

of the circulation, such as the mesenteric vascular bed. Therefore, results obtained from large conduit arteries from non-weight bearing limbs may not be indicative of changes associated with specific vascular beds, including the coronary and cerebral circulations.

In animal studies, spaceflight-induced apoptosis of vascular endothelial cells has been shown to occur in the retina and brain, and this loss of endothelial cells correspond with markers of disrupted barrier function of the blood-retinal[185] and blood-brain barriers[120,185,186]. Impairment of arterial endothelium-dependent vasodilator function has also been reported to occur in cerebral and mesenteric arteries following spaceflight[187,188]. HLU has been shown to diminish cerebral artery endothelium-dependent vasodilation through the eNOS signaling pathway[189] and possibly through an upregulation of vascular cell adhesion molecule-1[190].

Although animal studies demonstrate that spaceflight can have acute adverse effects on the vasculature, studies of mortality among US astronauts exposed to low-earth orbit, relative to the general population, non-astronaut NASA employees, and nonflight astronauts, indicate the risk of death due to cardiovascular disease is not elevated[191,192]. Thus, spaceflight appears to have few long-term adverse consequences on the cardiovascular system. These mortality data are, however, somewhat limited in that most astronauts included flew in space for relatively short periods of time and remained predominantly in low Earth orbit. The risk for developing cardiovascular disease may be elevated as mission duration increases or as exploration goes beyond the Earth's magnetosphere.

Space Radiation-induced Cardiovascular Response:

Emerging epidemiological research demonstrates that low-dose environmental, occupational, and medical radiation exposure increases the risk of mortality due to ischemic coronary artery and cerebrovascular disease[193,194]. However, these risk estimates of Earth-based irradiation are largely derived from low linear energy transfer radiation exposures, which have some fundamentally different properties from charged HZE particles present in the deep space environment. HZE ions, for example, produce greater adverse effects on cellular physiology through increased genetic alterations and perturbations to redox metabolism, leading to persistent elevations in oxidative stress[195]. Oxidative stress can impair vascular and cardiac function by direct oxidative damage or by activating cell signaling pathways that can lead to abnormal contractile, inflammatory, proliferative, or remodeling properties.

In animal studies, vascular endothelial cells have been shown to be especially sensitive to the effects of radiation. For example, Soucy et al.[196,197] demonstrated that simulated space irradiation with ^{56}Fe ions impaired endothelium-dependent vasodilation of the aorta and increased aortic stiffness; both these effects were secondary to the formation of reactive oxygen species. Other studies have likewise shown that simulated space radiation induces damage to vascular endothelial cells[198,199] which could lead to diminished barrier function and accelerated development of atherosclerotic plaque. Indeed, research by Yu et al.[200] has shown ^{56}Fe irradiated portions of the aorta in apolipoprotein E-deficient mice have accelerated atherogenesis in the targeted regions.

In addition, space radiation has an adverse impact on cardiac tissue. Low dose whole body irradiation with protons or ^{56}Fe ions were reported to produce myocardial DNA methylation[201]. ^{56}Fe irradiation has also been shown to diminish left ventricular function and increase infarction size and mortality rate in mice with a surgically induced myocardial infarction[202]. Collectively, these studies indicate that the damaging effects of space radiation can occur within the myocardium as well as the vasculature.

Combined Effects of Simulated Space Radiation and Weightlessness on Vascular Function:

The possible synergistic impact of space radiation combined with microgravity-induced weightlessness on degenerative cardiovascular disease in astronauts is poorly understood. To assess this possible synergy, Ghosh et al.[83] examined the single and combined effects of simulated space radiation with ^{56}Fe ions and simulated weightlessness using HLU on endothelium-dependent vasodilation of mouse gastrocnemius muscle resistance arteries soon after the cessation of the treatments. Both ^{56}Fe ion irradiation and HLU alone each impaired endothelium-dependent vasodilation, but this impairment was potentiated when the two treatments were combined. The endothelial dysfunction occurred primarily through the eNOS signaling pathway with ^{56}Fe ion irradiation and HLU, but with ^{56}Fe ion exposure the deficit was the apparent consequence of diminished anti-oxidant capacity and greater pro-oxidant protein expression in the artery, while with HLU the deficit in endothelium-dependent vasodilation was the apparent consequence of reduced eNOS protein content. These data indicate that a short-term consequence of space radiation exposure and weightlessness is the synergistic impairment of the vascular endothelium.

In a follow-up study to determine the long-term effects of simulated space radiation and weightlessness on vascular health, Delp et al.[192] repeated the abovementioned studies of Ghosh et al.[83], but gave the mice a 6–7 month recovery period, the human equivalent of 18–20 years, before the vascular studies were conducted. The results demonstrated that vascular impairment of endothelial function was not sustained in the HLU mice, which is consistent with the collective US astronaut cardiovascular mortality findings[191,192]. However, impairment of endothelium-dependent vasodilation persisted in the irradiated mice. This persistent radiation-induced endothelial dysfunction occurred through the eNOS signaling pathway and was associated with greater expression of the pro-oxidant protein xanthine oxidase. These data indicate that there are no long-term vascular consequences to weightless, but that the radiation effects of deep space travel on the vascular endothelium may endure through persistent elevations in oxidative stress[192].

Additional and Future Considerations:

Understanding of the singular and combined effects of space-associated weightlessness and irradiation is only now beginning to emerge, and many areas of research are open to further investigation. For example, vascular alterations induced by radiation may not be uniform throughout the circulation, given that the local biochemical milieu surrounding blood vessels and local mechanical forces (e.g., blood flow and shear stress) may interact with the local radiation effects to produce variable responses in different regions of the body, such as in the coronary and cerebral circulations. Much of what is known about the effects of spaceflight

on the cardiovascular system has come from the study of arterial vessels, but more research is needed to understand the effects of weightlessness and space radiation on venous and lymphatic vessels. This is particularly true given the likely involvement of venous and lymphatic vessels in the development of spaceflight associated neuro-ocular syndrome[115–118] and venous thrombosis[203,204] reported to occur in astronauts during long-duration spaceflight. Finally, little is known about possible sex-specific differences, or the effectiveness of various countermeasures (e.g., exercise, antioxidants, and nutraceuticals) on cardiovascular alterations associated with weightlessness and deep space irradiation. All these areas of research are vital as human exploration of deep space is being pursued.

6. STEM CELL RESPONSE

Tissue regeneration is both a highly mechanosensitive and radiosensitive process, relying heavily on mechanical cues imposed by Earth's gravity[205] and protection by the magnetosphere from harmful ionizing radiation. Data obtained from spaceflight experiments have documented the extensive effects of spaceflight stressors on progenitor cell populations and stem cell differentiation capabilities. These cellular changes often propagate into physiological defects which may pose significant health risks to astronauts both during and after sustained spaceflight missions. This section focuses on stem cell responses across physiologic systems, primarily through use of both ground-based HLU models, but also simulated microgravity devices (e.g., rotary cell culture systems, rotating wall vessels, and 3D clinostats).

The role of gravity in maintaining tissue homeostasis has become apparent with expanded study examining the effects of spaceflight on mammalian physiology. Dysfunction in stem cell populations contribute to many Earth-based disease conditions and can be enhanced by aging, oxidative stress, and genetic predisposition[206,207]. Adult stem cell populations are found in multiple physiological systems throughout the body and are surrounded by a highly organized and regulated microenvironment consisting of supporting cells and factors, resulting in the formation of a stem cell niche[208]. Following injury, damage, or normal cell attrition, stem cells within the niche receive signals resulting in transition to an active state and initiation of the differentiation process[209]. Therefore, in order for regeneration of damaged tissues to occur, resident stem cell pools must be activated and induced to differentiate into lineage specific cells[210]. Such activation signals may be biochemical or mechanical in nature, and therefore may be affected by exposure to microgravity. Spaceflight exposure may result in premature aging of specific physiological systems, and loss of stem cell functions may contribute to the initial observed tissue degeneration but more importantly, may be linked to regenerative deficits during long-duration spaceflight exposure beyond LEO.

Microgravity-induced Stem Cell Alterations:

Several studies specifically investigating the effects of microgravity on embryonic stem cell function have identified a deficit in differentiation capabilities in both true and simulated microgravity conditions[211,212]. Specifically, in spaceflight-induced microgravity mouse embryonic stem cells (ESCs) appear to maintain proliferative functions while failing to

express genes required for germ layer differentiation[212,213]. In simulated microgravity experiments with ESCs, results are ambiguous, with some studies reporting increased differentiation, while other studies report decreased adhesion and cell death. Parabolic flight is another method that has been used to simulate microgravity, with an added hypergravity component. Mouse ESCs exposed to parabolic flight showed significant alterations to gene expression, including cell proliferation, apoptosis and transforming growth factor-beta (TGF- β) signaling[214]. These cells also demonstrated increased differentiation into cardiomyocyte colonies following parabolic flight, as has been shown in several studies on the Space Shuttle[212,214].

Maintenance of stemness during microgravity exposure has also been demonstrated in several other stem cell populations including cardiovascular progenitor cells, mesenchymal stem cells (MSCs), hematopoietic (HSCs), and adipose derived stem cells. Specifically, studies using neonatal and adult cardiovascular progenitor cells exposed to microgravity on ISS and simulated microgravity in a clinostat resulted in altered cytoskeletal organization and migration in both cell populations[215,216]. Several of these responses were found to be regulated by miRNAs, thereby indicating that miRNAs may be a key mediator of the cellular response to spaceflight exposure[215–217]. MicroRNAs (miRNAs) are highly conserved non-coding RNA molecules that are involved in post-transcriptional regulation of gene expression. They function via base-pairing with complementary strands of mRNA, in turn silencing them by cleavage, destabilization, or hindering translation. Furthermore, the authors found reduced yes-associated protein 1 (YAP1) and Tafazzin (TAZ) signaling that can function to regulate transcription and is affected by mechanical load[217]. Neonatal but not adult cardiovascular progenitor cells exposed to spaceflight exhibited increased expression of markers for early cardiovascular development and enhanced proliferative potential, possibly mediated through miRNA signaling[215,216].

YAP and TAZ signaling also play an important role in MSCs, resulting in the expression of Runx2 and initiation of osteogenesis. The effect of microgravity on MSCs has been widely studied. Studies investigating YAP and TAZ signaling under simulated microgravity have shown inhibition of osteogenic differentiation by MSCs due to lack of TAZ nuclear translocation[218,219].

Some of the most well-documented changes to MSCs are morphological and include alterations to the cytoskeletal architecture, adhesion properties and migration patterns. In simulated microgravity studies, hMSCs were found to be flatter with disruption of the actin cytoskeleton, redistribution for vinculin and increased integrin expression[220,221]. Some reports have indicated increased proliferation of hMSCs cultured under simulated microgravity while others have reported decreased proliferation with reduced expression of cell cycle related genes during simulated microgravity and true spaceflight exposure[222,223]. Furthermore, hMSC cultures subjected to unloading via clinorotation have been shown to exhibit preservation of their dedifferentiated state as well as successful trans-differentiation[222,224]. These studies highlight the differences between culture systems and the need for standardized methods to assess the role of microgravity on cell and stem cell functions.

Several investigators have confirmed the inhibition of stem cell differentiation *in vivo*, as expressed in the osteochondrogenic precursor stem cell lineage in mouse models[225]. Mice exposed to spaceflight on the BionM1 capsule demonstrated reduced MSC commitment and corresponding increase in differentiation of the MSC lineage into osteoblasts and adipocytes following reloading[226]. Further experimentation of microgravity-induced altered gene expression suggests miR-132–3p as a potential inhibitor of osteoblastic differentiation[225]. Induction of cellular senescence is another potential contributor to the permanent proliferative state incurred by many cell populations under microgravity simulations. Minimal research has been conducted to elucidate the specific molecular modulators involved in spaceflight-induced cellular senescence; however transcriptomic analysis of space-flown mice suggests a p53-independent induction of the p21 senescence pathway[227]. Nevertheless, further experimentation is needed to fully elucidate these molecular pathways. Another interesting phenomenon is the change in lineage commitment of MSCs from the osteogenic lineage to adipogenic lineages similar to that which occurs during aging[228]. Several studies conducted using directed differentiation of MSCs under simulated microgravity have shown a preferential commitment to the adipogenic lineage through a variety of mechanisms including suppression of microfilament formation and RhoA activity[229].

The hematopoietic system is of critical importance for immune function and red blood cell production, while also contributing to the maintenance of the skeletal system. Several studies have investigated alterations to the immune system and indicated that spaceflight results in shifts in immune cell phenotypes, reduction in circulating T cells, and decreased T cell activation, decrease in B cell numbers, and decreased numbers and cytotoxicity of NK cells[230–233]. As HSCs are the primary contributors of erythropoiesis, myelopoiesis, and lymphopoiesis[234], it is critical to understand the effects of microgravity on basic HSC function and correlate these changes to altered immune functions observed during spaceflight.

Studies investigating hematopoietic lineage cells in astronauts found decreases in NK cells and reticulocytes following short duration spaceflight[234]. Post-flight assessment of astronauts indicate deficiencies in thymopoiesis[235], a reduction in erythroid and myeloid progenitor cells[236], and altered whole-blood transcriptomic and metabolomic profiles[237]. Interestingly HLU studies found significant changes to bone marrow HSC populations including increases in long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), multipotent progenitor (MPP) cells and neutrophils but observed decreases in B-lineage cells, NK cells and erythrocytes. No recovery in HSCs and neutrophils following a 28-d reloading period was observed and repopulation assays showed significant impairment of the reconstitution ability of HSCs exposed to HLU[234]. Interestingly, significant alterations in myeloid colony forming units was also observed in mice following exposure to spaceflight on the BionM1 capsule[238]. Mice flown on the BionM1 capsule demonstrated a decrease in hematopoietic cells and a suppression of primitive multipotent progenitors that were not found to be reversible during a 7-day recovery period[226]. Human HSCs exposed to simulated microgravity have shown impairment of DNA damage repair mechanisms and accumulation of double stranded DNA breaks[239]. The differentiation capacity of these cells into dendritic cells was also impaired[239]. These studies highlight the reduced hematopoietic functions of

HSCs following spaceflight and may demonstrate that altered immune responses in response to spaceflight exposure may originate, in part from deficits to HSC function.

The effects of microgravity on neural precursor cells has also been gaining interest in recent years. Several studies have been conducted using simulated microgravity, including differentiation studies investigating the role of microgravity on human ESC-derived neural organoids[240]. Organoids exposed to simulated microgravity exhibited deficits in expression of forebrain markers indicating that microgravity directs differentiation towards caudal neural progenitors[240]. Interesting, as demonstrated in other stem cell populations, Wnt signaling may be a key signaling mechanism involved in this response[240].

Overall, studies investigating the impact of microgravity on stem cell function suggest that microgravity critically affects basic cell functions including alterations to cell morphology, cytoskeletal organization, gene expression profiles, differentiation and apoptosis related pathways[218,223,232,241,242], contributing to the growing concern that the health of both stem cells and terminally differentiated lineage populations is threatened during spaceflight exposure. Findings that highlight the inability of stem cells to differentiate towards certain lineages emphasize the detrimental effect of microgravity on tissue regenerative processes and the need to develop therapeutic countermeasures before long-duration spaceflight missions are attempted.

Radiation-induced Stem Cell Dysfunction:

The effect of cosmic radiation on stem cell function is not well understood and studies have focused primarily on MSC/HSC populations. Multiple studies demonstrate a notable impact on primitive and differentiated hematopoietic cell lines after GCR/solar energetic particles (SEP) radiation exposure[243,244]. The progenitors of erythroid, T-lymphocytes, and B-lymphocyte lineages were seen to demonstrate radiosensitivity[244], highlighting the potential of radiation exposure to destabilize immune function and efficiency. Whole-body blood cell counts after proton radiation confirm these findings indicating decreased amounts of white blood cells, lymphocytes, and neutrophils up to 100 days post-irradiation[244]. Additionally, HSCs may be affected through their corresponding microenvironment, influenced by adjacent irradiated cell populations through the bystander effect. Coculture experiments of unirradiated HSC and irradiated MSC mixtures show that HSC populations experience indirect effects of GCR/SEP radiation of MSCs along with the previously established direct effects resulting from HSC irradiation[243]. These results clearly display the concept that microenvironmental changes in the bone marrow induced by radiation exposure significantly impact bone marrow progenitor cells both directly and indirectly.

Several studies investigated the neurogenic risks of simulated space radiation environments, emphasizing a significant decrease in neural stem and progenitor cells[245,246]. Neurogenesis is required for cognitive strength, mood regulation, and neurological stability, thereby stressing the importance of characterizing any cosmic radiation-induced effects on neural stem cells (NSCs)[247]. While both NSCs and proliferating neural progenitor cells were negatively affected by cosmic radiation, quiescent NSCs were found to be particularly vulnerable compared to their rapidly dividing progeny[245]. This suggests that mechanisms unrelated to cellular replication may be impacted by GCR, such as oxidative stress, and

implies long-term negative effects to stem cell populations and their differentiated progeny. HZE of GCR are shown to be directly associated with mitochondrial dysfunction and ROS production, causing damage and death of affected cells[247]. NSCs in the hippocampal region may be especially susceptible to cosmic radiation-induced oxidative damage, attenuating neurogenesis and impairing cognitive function. Furthermore, murine cerebral exposure to heavy ion radiation with high linear energy transfer (high-LET) results in a premature aging phenotype through accelerated p21-mediated neuronal cell senescence[246]. This phenomenon is also evident among other somatic stem cells, including endothelial progenitor cells, osteochondral progenitor cells, and lung progenitor cells[248]. These findings report the neurogenic risks of spaceflight radiation exposure and identify the need for further research into adult stem cell populations and the propagated tissue-based effects.

GCR-induced alterations in ESC functions have not been studied in great detail. A genomic analysis of murine ESCs during spaceflight reveals suppression of global gene expression and increased spontaneous chromosomal aberrations[249]. These genomic defects may contribute to increased ESC dysfunction or reprogramming. Furthermore, since ESCs are vital for repairing diseased tissue, loss of ESC stemness or differentiation abilities can adversely impact tissue regeneration and wound healing.

Combined Effects of Radiation and Microgravity on Stem Cells:

Stem cell regulation has been defined for individual spaceflight stressors (i.e. microgravity and radiation); however one of the fundamental questions to be answered in regenerative space biology is whether these stressors have synergistic effects on progenitor populations. Several studies describing tissue-level compounded effects by microgravity and radiation in the brain, retina, and skeleton have been reported[250,251]. It is possible that these tissue-based effects stem from aberrations in progenitor cell populations. Research conducted using gamma and heavy ion radiation demonstrated deficits in osteoblastogenesis due to increased oxidative stress in progenitor populations[78,96,252]. Additionally, as previously noted in Section 2, combined exposure of mice to HLU and radiation demonstrated depletion of progenitor populations that were rescued by administration of an antioxidant rich diet, the dried plum diet[94]. Recent research details that microgravity-induced osteoclast progenitor cell counts are modulated by space-standardized radiation[251].

Other studies investigate the compounded effects of microgravity and proton irradiation on immune function and leukocyte activity. Lymphocytes are considered one of the most sensitive mammalian blood cell types to ionizing radiation exposure; thus it is important to characterize any microgravity-associated augmentation to this sensitivity. One study utilized the HLU model and solar particle event-like proton radiation to conclude that spaceflight factors do have a synergistic effect on the proliferation rate and activation rate of isolated splenic T lymphocytes. They found that combining HLU with proton irradiation significantly suppresses splenic T lymphocyte activation 21 days post exposure while diminishing the proliferation rate[77]. However, results are controversial, as another study reports no interplay between radiation and rotating wall vessel-simulated microgravity in human lymphocytes[253].

Several research groups suggest that miRNAs may contribute to the regulation of compounded spaceflight effects[254]. Certain miRNAs may be differentially expressed depending on the combination of spaceflight factors inflicted. One such group demonstrates this by analyzing miRNA transcription profiles in peripheral blood lymphocytes exposed to simulated spaceflight. They conclude that simulated microgravity modulated the effect of radiation exposure by downregulating radiosensitive miRNAs, including important promoters of osteoblastic stem cell differentiation (miRNA-144), iPSC generation (miRNA-200a), and embryonic stem cell differentiation (miRNA-7)[255]. Lastly, research involving the effects of radiation and microgravity on human lymphoblastoid TK6 cells reveal no differentially expressed miRNAs under isolated factors. However, an interactive effect between the spaceflight conditions is exhibited, resulting in differentially expressed miRNA-15b and miRNA-221, both of which promotes stemness and tumorgenicity of cancer cells[256]. Taken together, research involving the interplay of radiation and spaceflight in stem cell functionality indicates a synergistic relationship, compounding the effects seen by each isolated factor.

Additional and Future Considerations:

Due to the extensive applications and potential benefits offered by stem cells therapies, research into these populations has increased over the past 10 years; however, we have only recently begun characterizing the effect of spaceflight stressors on stem cell populations and tissue regeneration. A majority of these studies have investigated alterations to HSCs or MSCs, leaving studies into tissue-specific and ESCs during spaceflight lacking[257]. Additionally, availability of true-spaceflight research focused on stem cell populations is limited due to limited resources and technology, therefore as noted researches must rely on ground-based models. As noted, these models fail to encompass all of the stressors and physiological effects associated with true-spaceflight, including full-body mechanical unloading, changes in fluid dynamics and systemic alterations[258]. As a result, research conducted to establish the compounded effects of microgravity, radiation, and other spaceflight-associated environmental changes on stem cell function are needed.

Recent developments in research programs led by the ISS National Laboratory in collaboration with National Institutes of Health (NIH) and National Science Foundation (NSF), have expanded the capacity for stem cell research to be conducted in space. Specifically, these programs have increased interest in Earth-based applications and have specifically called for the use of micro-physiological systems and organ-based cultures in space. These programs, and foundational studies characterizing the maintenance of stem cell properties in populations exposed to microgravity conditions, have presented a unique opportunity for the use of stem cell populations cultured under microgravity conditions for tissue engineering and regenerative medicine applications. This has been explored in multiple paradigms, including the use of cranial-derived MSCs as a potential cell therapy for the CNS[259], and is reviewed extensively elsewhere[258,260]. Although stem cells have been shown in multiple studies to retain their stem cell characteristics and exhibit delayed differentiation, there are also many other changes that have been observed related to microgravity exposure[261,262]. These alterations may result in unintended consequences from using stem cell populations cultured under microgravity conditions in cell-based therapies.

As we start to explore the Solar System beyond LEO, from the Moon to Mars, expansion of studies in LEO investigating the effects of microgravity on stem cell function to the combined effects of microgravity and space radiation will be critical for understanding tissue regeneration capabilities. Maintenance of stemness in microgravity also poses a significant risk for the maintenance of tissue homeostasis, and for tissue repair and regeneration during long-duration spaceflight exposure. Exposure of radio-sensitive stem cell populations to deep-space radiation poses a significant concern for permanent and irreversible damage to stem cell populations while also posing a risk for stem cell depletion. Accumulation of DNA damage in stem cell populations that are activated upon stimulation, is also a cause for concern. However, in-depth research into the effects of combined spaceflight stressors on the numerous stem cell populations found in mammalian organisms is lacking. Furthermore, studies using whole animals and human cell populations (such as iPSCs) are also lacking; resulting in an incomplete picture of the effects of combined spaceflight stressors on stem cell populations. Although several studies have touched on the molecular mechanisms driving the observed changes, an in-depth understanding of the local and systemic mechanisms are still to be elucidated. Therefore, as new opportunities for spaceflight experiments are explored, it is critical to pursue the use of spaceflight for Earth-based benefits (including understanding basic stem cell function in addition to exploration of clinical applications) in addition to research enabling human exploration.

7. CONCLUSIONS AND ADDITIONAL CONSIDERATIONS REGARDING THE HLU MODEL

In LEO, the influence of microgravity on physiological systems is the most prominent spaceflight factor. Due to limited access to space, many studies have been conducted utilizing models of microgravity, including HLU in rodents, and simulation devices based on randomizing the gravity vector, or subjecting cell masses to consistent free fall conditions. Specific analysis of simulation devices is beyond the scope of this review; however, it is important to note that the utility of these devices to simulate microgravity is still debated[223], partially due to the question of whether individual mammalian cells have the ability to sense gravity, or whether they sense their mechanical environment (which is altered by gravity) instead. Although these methods of simulating microgravity during spaceflight present unique drawbacks and limitations, they all generate results indicating that microgravity-facilitated mechanical unloading alters stem cell behavior and activity. This highlights the need to continue, and expand upon, studies focused on basic stem cell function under true microgravity conditions, in order to elucidate the potential physiological effects that these changes may have during long-duration spaceflight[212,263–265].

Limitations exist regarding the use of HLU. Hind limb unloading via tail suspension is a good instrument to study bone demineralization associated with microgravity in rats and mice. In rats, HLU leads to many of the cardiovascular changes that occur in humans during spaceflight, including the cephalic fluid shift[266,267]. HLU invokes many of the changes in blood vessel structure and region-specific changes in blood flow that are seen in rats during space flight[189,268] and also results in changes in the ultrastructure of the choroid plexus and CSF production that closely resemble those seen in rats that have been in space flight[269,270].

Factors such as altered neuroendocrine functions, behavioral deficits, and increased stress levels[271,272] should be considered when designing studies and interpreting results. Stress is clearly a feature that astronauts will face during spaceflight), though astronauts typically have a high tolerance for stress. Porphyria, which is a sign of distress in laboratory rodents[273] is a common, though transient, trait of rodents during HLU procedure[28]. HLU models can increase stress response as indicated by elevated serum corticosterone that is associated with atrophy of lymphoid organs[36,274–276], although these responses are not always consistent. A primary technical report detailing aspects regarding HLU[28] recommends housing single rodents at between 24.5C-25.5°C to mitigate any toxicity profiles (e.g., bone loss, impaired ability to thermoregulate[31,277]). Moreover, based on experience of all of the authors, attrition of rats from HLU studies (e.g., sudden loss of rodents during the study, excessive porphyria, or persistent weight loss) occurs more frequently if housing is below this temperature range.

With a few exceptions[34,80], studies involving HLU generally involve isolating rodents via single housing because of jig constraints[35,278], which could impact immune or stress responses[36]. Moreover, single housing in social species like rodents can profoundly alter physiology. For example, isolation can lead to anxiety[279], impairments in memory[280], upregulation of neuroendocrine responses to stress[281–283284] and altered immunity[285,286]. As flight studies typically involve group housed mice vs single housed conditions, one group has performed a side by side comparison of HLU under single and socially-housed conditions[36]. Results from this study reveal that deficits in skeletal structure typically attributed to HLU generally occur at a similar magnitude in single and socially-housed animals. This suggests that bone loss as observed in the HLU model results primarily from gravity-dependent mechanisms. In contrast, some immune system responses to HLU are differentially impacted by the social environment. Moreover, isolation impacts neural systems that mediate mission critical cognitive domains. For example, isolation has been observed to reduce makers of neuroplasticity in the rodent hippocampus and prefrontal cortex[287–289]. In addition, isolation has been observed to increase markers of neuroinflammation and cell loss in these brain structures[287,290,291], increases redox stress, proinflammatory tumor necrosis factor α (TNFα) levels (in the hippocampus)[290], and reduces Parvalbumin (PV)+ interneurons in CA2 and CA3[292]. Thus, factors such as room temperature, social housing, and stress responses should be considered when performing and interpreting individual and combined hazard investigations, such as HLU + space radiation.

While there are many facsimiles of flight stressors that can be used in rodents to mimic the neurological and psychological impacts seen in humans, modeling microgravity effects on the CNS using HLU is problematic. As noted, microgravity is documented to impact many aspects of mammalian physiology, including the skeletal, microbiome, gut mucosa, sensorimotor and ocular systems (e.g.,[28,76,117,293–295]). All of these are likely to have some to major impacts on the brain, either directly or indirectly, and interact with the sequelae from other flight stressors. Obviously, with accepted limitations, there are no ground-based systems that can be used on rodents to fully simulate microgravity. As noted, simulated microgravity[145] results in persistent changes in the mitochondrial function and lipid metabolism within oligodendrocytes. While clinostats seem to do a reasonable job of

simulating microgravity effects in oligodendrocytes[145] no such devices are readily available for rodents.

Thus while HLU may induce some of the physical and phenotypic changes associated with space flight, it remains unclear if HLU induces similar signaling and molecular changes (e.g. increase in fatty acid metabolism that will impact neuronal function and thus cognition) as with microgravity. Moreover, whatever responses are induced as a result of the HLU, these occur in an animal that can be assumed to be under stress – although this in itself is a characteristic response in astronauts during spaceflight. The impact of such stress may invoke compensatory mechanisms that may, or may not, occur in humans while in space. Thus as noted, limitations should be considered with use of the HLU model. While PWB represents an alternative model and offers the possibility to investigate a wider range of mechanical loads, more studies are needed to investigate the response of multiple organ systems to reduced weight-bearing. For example, radiations have only been administered in PWB female mice, and more studies are needed to assess the benefits of this model to radiation research in larger outbred rodents. Further studies should also more completely profile the stress response resulting from PWB.

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