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Changes in Mice Calvaria Following Fifteen Days in Space

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by

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The thesis of Roshmi Bhattacharya is approved and it is acceptable in quality and form for publication on microfilm:

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ABSTRACT OF THE THESIS

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Physiological changes in response to microgravity are some of the main concerns that must be taken into account prior to a space mission. Bone remodeling may occur in spaceflight as a response to unloading of the lower limbs and head-ward fluid shifts. While unloading results in significant loss of bone mass and density in the lower limbs of animals exposed to microgravity, increased fluid flow to the head may elicit the opposite effect. In bones that normally do not bear weight such as the skull, it has been hypothesized that adaptation to microgravity can induce growth. This paper discusses the

various physiological effects of microgravity on astronauts and new data on remodeling of the skull in space. Mice were sent to space on the 15-day STS-131 mission, and upon their return, characteristics of their calvaria and those of ground controls were evaluated by micro-computed tomography (micro-CT) and biomechanical analysis. Micro-CT analysis indicated significantly greater bone volume over total volume in the spaceflight group, 1.904 ± 0.842 mm^3, compared to 1.758 ± 0.122 mm^3 for that of the control group ($p<0.05$). Likely due to the short duration of spaceflight, there was no significant difference in the other parameters, cortical thickness and tissue mineral density. Micro-indentation was conducted on the calvaria to determine stiffness. Taken over several consistent points on each specimen, the elastic modulus in the spaceflight group was significantly greater, 10.5 ± 1.9 GPa, compared to 9.3 ± 2.1 GPa in the control group. From this, we concluded that exposure to microgravity causes adaptive growth in calvarial bones.

Introduction

Long-duration spaceflight causes significant physiological changes in astronauts as a consequence of the hypothesized fluid shift caused by microgravity. In space, as blood flow redistributes away from the feet toward the head, intracranial pressure increases leading to elevated intraocular pressure and potential vision impairment. However, limited research has been carried out in space to study these changes.

The impracticability of conducting repeated large-scale experiments and carrying the necessary equipment on space shuttles restricts research in spaceflight. However, bed rest is an established ground model for simulated microgravity and investigation for the effects of the fluid shift. In this protocol, subjects are positioned in a supine or head-down tilt position continuously for a number of days. A study by Leblanc and colleagues (2000) includes a 120-day bed rest cohort that exhibits significant although less pronounced percent changes in bone mineral density (BMD) of the spine, femur, pelvis, arm, and leg and lean muscle tissue of the arm and leg compared to the astronaut group. The lower rates may be attributed to the shorter duration of bed rest compared to the variable 4-month to 14.4-month duration of the spaceflight group. A 60-day bed-rest study reports the most loss at the proximal femur and distal tibia, consistent with the conclusions reported in spaceflight that the lower limbs are affected to the greatest extent (Beller et al., 2011).

The head-down tilt position is a commonly used method in bed rest studies in order to induce a larger head-ward fluid shift and achieve the drastic results experienced in spaceflight while allowing for shorter duration studies (Yamasaki & Shimizu, 2000). The correlation between disuse and bone loss in the lower limbs has also been established

with hind limb unloading in the mouse model. Hind limb unloading involves suspension of only the lower limbs often by attaching a ring around the tail of the mouse to a rope tied to the top of the cage. Initially and during continued unloading, femur and tibia perfusion decreased (Colleran et al., 2000). Mass was significantly lower in these bones in the experimental group, which had experienced 28 days of unloading as compared to the control group with 11% difference in the femur and 6% in the tibia. The opposite effect was seen in non-weight-bearing bones of the upper body such as the mandible, clavicle, and humerus with increases of 10%, 18%, and 8%, respectively, between the groups. However, the field currently lacks comparable data on remodeling in microgravity of normally unloaded bones such as the skull, which our study aims to address.

Cardiovascular deconditioning presents serious challenges to astronauts during and after spaceflights. The zero gravity environment causes the human cardiovascular system and other body systems to undergo both functional and structural adaptive alterations, impairing regulatory functions which normally sustain the stability of the human cardiovascular system on Earth (Whedon, 1978; van Loon, 1996). The cardiovascular system is designed to carry blood up against gravity to reach the upper body and prevent pooling in our lower limbs. Microgravity leads to the loss of these blood pressure gradients, causing an upward fluid shift, which leads to facial edema, headaches, and nasal congestion (Hamilton et al., 2012). This triggers receptors in the upper body to perceive it as excess fluid and works eliminates it, causing increased urination and decreased total body water by approximately 2-3% and leading to a decrease in blood plasma by approximately 10-20%, leading to hypovolemia (Convertino

1995). Ventricular remodeling may contribute to decreased stroke volume and causes several other cardiovascular effects, such as increases in the filling pressure of the left ventricle and pulmonary venous congestion, as well as decreased cardiac output causing symptoms of congestive heart failure (Perhonen et al., 2001; Hoit, 1991). Hughson et al. (2013) have found that gravitational force changes, modifications in physical activity patterns, and social factors may also cause accelerated the stiffening of blood vessels along with the development of atherosclerosis, similar to aging.

Soviet researchers have recorded a constant increase in pressure in the jugular veins and a decline in pressure in the leg venous vessels during space missions. The scientists noted that during the 185th day of the Salyut-6-Soyuz mission, the average volume of the lower parts of the body of the two astronauts stood at only 10 percent (Genin & Egorov, 1981), showing that in the lower extremities, a combination of disuse and decreased arterial pressure results in muscle atrophy and loss in bone mineral density. Recent research aims to mitigate these effects, especially through the use of countermeasures such as lower body negative pressure (LBNP), aerobic exercise and devices (ex. “thigh cuffs”) that are designed to maintain pressure in the lower limbs in order to promote physical health throughout and after extended exposure to microgravity (Aratow et al., 1993; Trappe at al., 2009).

Spaceflight countermeasures are methods that are being investigated as part of a protocol to mitigate the effects of spaceflight. For several years, astronauts have practiced physical fitness while in zero gravity through the use of bungee cord restrictive exercises together with treadmill exercises, aerobics and bicycle ergometer (Nicogossian et al., 1994: Shackelford et al., 2004; Bioastronautics, 2003). However, exercise alone has been

inadequate at preventing these changes; therefore, using it in conjunction with countermeasures that combat this redistribution of fluid will allow for the maintenance of bone and muscle, significantly decreasing the risk of injury in astronauts during and after spaceflight. The short-arm centrifuge in the development of the gravity forces is likely to triumph over the numerous probable negative side effects such as dizziness, effects on both cognitive and motor functions, and motion sickness. On the other hand the use of the long-arm centrifuge would be advantageous in preventing some of these side effects but would be disadvantageous in terms of its cost, mass and size (Bronner et al., 2012). Another technique is lower body negative pressure (LBNP) in which negative pressure is applied within a chamber that is sealed below the waist to prevent the head-ward fluid shift (Aratow et al., 1993). It is theorized to do this by increasing the interstitial fluid pressure with constant capillary pressure generating a Starling force that drives fluid flow into the tissues of the lower limb. Short-term data shows a decrease in plasma volume and increase in leg circumference that supports this mechanism. Interstitial fluid pressures were elevated in parallel with chamber pressure during the study but showed no lasting results afterward. LBNP combined with treadmill exercise has also been used to maintain plasma volume, orthostatic tolerance, upright exercise capacity, as well as muscle strength and endurance during 30 days of head-down tilt bed rest (Hargens 2009). However, in order to develop an effective countermeasure protocol, it is imperative to better understand processes such as bone remodeling during spaceflight.

The fundamental structural unit of the bone is a hollow collagen rod and calcium phosphate. At the bone shaft are several rods that are bundled together and positioned in a compact bone ring, forming a strong cortical shell. The main function of the shell is to

offer optimal resistance from bending and to provide the necessary protection to the bone marrow. A network of trabecular bones that are also referred to as spongy or cancellous bones are found towards the end of the bone, recording the highest amount of loss of bone mineral density in space (Carlsson et al., 2003). Human bones have been noted to persistently undergo both the growth and resorption processes. The mineralization, which is the laying down of new bones, is carried out by osteoblasts while resorption is carried out by osteoclasts. Zero gravity has been shown to cause slower rates of mineralization, inhibition of osteoblasts and a higher rate of resorption (Carlsson et al, 2003).

On Earth, the human body absorbs a total of between 40 and 50 % of the total calcium intake while under zero gravity the body's calcium intake declines to between 20 and 25% (Zhao et al., 2010). Approximately 250 mg of bone calcium is lost per day of spaceflight, while astronauts regain bone at a much slower rate of 100 mg of bone calcium per day for the first 3 months post-flight (Smith et al., 1999). During spaceflight, the combination of microgravity, high carbon dioxide concentration and dim lighting have been shown to adversely affect the skeletal system, resulting in a bone loss of up to two percent per month (Bruce 2002). Bone loss experienced during weightlessness mostly affects the load-bearing bones, including the lower lumbar vertebrae, pelvis, tibia, ankles, femoral neck and greater trochanter (Buckey 2006). It is imperative to study bone remodeling during weightlessness in order to understand the skeletal changes associated with the fluid shift and develop means to maintain bone density. Bone depletion is caused by a number of factors, including reduction in blood pressure to the legs and significant deficiencies in vitamins and minerals that are necessary for bone maintenance. Iwamoto, Takeda & Sato (2005) conclude that weightlessness results in deficiencies in calcium,

vitamin D and vitamin K, along with increased urinary calcium output and thus increased risk of kidney stones, decreased intestinal calcium absorption, reduced serum parathyroid hormone and calcitriol, and increased serum calcium level. There is a loss of 1.0-1.6% BMD per month in the spine, femoral neck and trochanter, and pelvis. They report that the efficiency of pharmaceutical agents has not been supported in the data on humans while hind-limb suspension studies of rats show preventative effects of vitamin K2, testosterone and bisphosphonates on BMD loss in the hind limbs and cancellous bone loss in the tibia. However, there is uncertainty about cortical bone loss prevention. Exercise-based treatments are used to reduce bone loss, as well as prevent atrophy of the muscles and heart and decrease cardiovascular deconditioning (Barry, 2001).

NASA, in its Bioastronautics Critical Path Roadmap for space associated health issues, formally terms the space bone density loss occurrence as a "zero gravity stimulated acceleration of age linked osteoporosis". Nonetheless, current research on bone density loss experienced by astronauts compared to the data on osteoporosis shows that the mechanism of the zero gravity stimulated bone density loss and age-linked osteoporosis vary. In comparison to the heightened rate of bone resorption, which is one of the traits of osteoporosis, the hypothesis of bone density loss in the lower limbs in space is that bone loss occurs as a result of the inhibition of the osteoblastic activities, thereby decreasing mineralization while bone resorption rates are at or above normal (Convertino et al., 1989).

A study comparing BMD of the spine, femur, pelvis, arm, and leg as well as lean muscle tissue in the arm and leg before and after spaceflight found significant rates of decrease in both parameters in all body parts except in the arm (LeBlanc et al., 2000).

The astronauts exhibited high rates of loss in BMD up to 1.56% per month in the trochanter, the upper part of the femur, and lean muscle tissue up to 1% per month in the leg despite engaging in a structured exercise program. In addition, bone measurements on Skylab astronauts show increasing mineral loss in the os calcis, also known as the calcaneus, corresponding to the length of the mission, raising the concern that BMD loss may not reach a threshold and continues throughout the duration of spaceflight (Smith et al., 1977). Restoration of BMD is a very slow process and may take several years post-spaceflight depending on the person, indicating that osteoporosis and risk of fracture of the normally weight-bearing bones of the body upon return to Earth may be a long-term or even permanent problem. These negative effects also compromise astronaut safety during extravehicular activities such as maintenance of the space station and other daily tasks in microgravity.

Current research focuses on cardiovascular changes as well as skeletal changes in the lower limbs. The purpose of this study is to present novel data about bone remodeling in the skull of mice in space, as there is a significant lack of research and information in this area. It also aims to validate ground studies regarding the effects of the fluid shift on bone and present new information that will be relevant for future studies regarding in astronauts during spaceflight.

Materials and Methods

Experimental protocol conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health, and was approved by the Institutional and Animal Care and Use Committee of the National Aeronautics and Space Administration (NASA). Prior to experiments, all animals were deemed healthy by the Kennedy Space Center (KSC) veterinarians. Seven 23-week-old adult, female, congenic C57BL/6 mice representing the spaceflight group (n=7) experienced fifteen days of microgravity aboard the 15-day NASA shuttle mission STS-131. Eight female congenic C57BL/6 mice, littermates of the spaceflight group, were maintained on land under normal gravitational loads and represent the control group (n=8). Both groups were maintained on a 12:12-hour lightdark cycle and provided with pre-adapted food and water ad libitum, from April 4 to 20, 2010 for the flight mice and from April 6 to 22, 2010 for the ground controls. Both groups were housed in Animal Enclosure Modules with gravity or lack thereof being the primary environmental difference. All mice were weighed twice – once just prior to cage-loading, and again after cage-unloading at the termination of the 15-day period and prior to sacrifice, and the weight change of each animal was determined. After mission completion, the spaceflight mice were received and euthanized at the NASA Kennedy Space Center, and their tissues were harvested within three to four hours of shuttle landing. The ground control mice were identically housed for the duration of the STS-131 mission beginning 48 hours after launch and were euthanized at the termination of their experimental duration 48 hours after shuttle landing. All animals were handled and dissected by members of the Biospecimen Sharing Project at NASAKSC. The calvariae were frozen in liquid nitrogen

and shipped under dry ice to our laboratory at University of California, San Diego for analysis. It should be noted that the STS-131 mission originally carried eleven adult, female, congenic C57BL/6 mice. However, only seven were included in the current study. One calvaria suffered multiple bone fractures from inappropriate handling and storage, and was deemed unsuitable for analysis. Three were euthanized twenty-eight hours after landing instead of within four hours, and thus were not included in our analysis. Although the addition of these three calvariae would greatly increase the sample size, we felt that since the length of stay in microgravity was only fifteen days, the three calvariae extracted after experiencing Earth's gravity for more than one entire day may not truly reflect microgravity-induced changes and may instead introduce bias to the analysis of the samples. To compensate for this decision, we also excluded three of the eleven ground control mice with the same number of caged-days prior to euthanasia.

Samples were thawed and imaged on a micro-Computed Tomography scanner, Skyscan 1076 (Kontich, Belgium). Calvariae were wrapped in tissue paper moistened with Phosphate Buffered Saline (PBS) and scanned at 9μm voxel size, applying an electrical potential of 50 kVp and current of 200uA, and using a 0.5mm aluminum filter. Tissue Mineral Density (TMD) was determined by calibration of images against 2mm diameter hydroxyapatite (HA) rods (250 and 750 mg/cm3) with a beam hardening correction algorithm applied during image reconstruction. Bone structure and image analysis were visualized and performed using Skyscan software, Dataviewer, CTAn (Kontich, Belgium). A volume of interest (VOI, defined by a standardized rectangular volume manually sized by computer cursor to measure 18mm3, 5.5mm x 1.2mm area in

the coronal plane, 2.7mm depth) identical for every calvaria was located at the center of the parietal bones (Fig. 1, panels A, G and H) by identifying the intersection of the anterior lambdoid and coronal sutures with the sagittal suture to position the center of the VOI on the sagittal plane and localizing the VOI center on the coronal plane at the sagittal suture. After applying a global threshold, an erosion of one pixel was performed to eliminate partial volume effects. The following parameters were determined using the aforementioned micro-CT software: bone volume (BV, defined as the volume within the VOI occupied by bone), average cortical thickness (Ct.Th, measured as the average thickness of the calvaria from the edge of cortical bone underlying the scalp to overlying the meninges, calculated in 2D on the basis of the plate model ("Morphometric Parameters in CT-Analyser"), and tissue mineral density (TMD, determined by calibration of the attenuation values to those obtained from the HA rods). Mathematically, the Ct.Th was calculated by first determining the area of the sub-pericranial surface (mm2) of the calvaria that's enclosed by the VOI, and then dividing the BV (mm3) by this area to reach a value expressed in millimeters. The TMD was computed by first obtaining the bone mineral content (grams) within the BV (cc), and then subsequently dividing bone mineral content by the BV to yield grams/cc. Therefore, the TMD is a true representation of the average mineral content within each cc of BV, and is independent of the amount of non-bony material within the VOI or the vertical dimension of the VOI (Fig. 1, panel G). Bouxsein et al. defined the standard for minimal set of reportable variables when describing cortical bone morphology with micro-CT: total cross-sectional area inside the periosteal envelope (Tt.Ar), cortical bone area (Ct.Ar = cortical volume (Ct.V) / (number of slices * slice thickness)), cortical area fraction

(Ct.Ar/Tt.Ar), and average cortical thickness (Ct.Th). However, since these guidelines are intended for reporting micro-CT values derived from a cylindrical VOI containing both cortical and trabecular bone (ex: a femur), the aforementioned reportable variables do not fully apply to our samples, which are solely composed of cortical bone. Specifically, Tt.Ar is not applicable to calvaria, and Ct.Ar/Tt.Ar would yield a value of 1 for all the samples. We chose to present the BV (equivalent to the Ct.V because the calvaria is entirely cortical bone) instead of the Ct.Ar because it is a more accurate representation of the differences between the spaceflight and control groups. Also, calvarial bone is not present in all slices taken by the micro-CT within the VOI (Fig. 1, panel G), unlike that expected when the VOI is localized length-wise to the mid-section of a long bone. Finally, the Ct.Th is an appropriate variable and is reported accordingly. Computations were performed with SPSS. The effect of spaceflight relative to ground control was assessed by unpaired two-tailed t-tests for the parameters of weight change, BV, Ct.Th and TMD, respectively. Significance was set at $p < 0.05$. Results are presented as mean ± SD in text and Table 1. For graphical clarity, data are presented as mean ± SE in Figure 3.

These calvariae were later assessed biomechanically using a microindentation technique to determine the local compressive modulus of calvaria flat bone. The microindentation was conducted in the following manner: Calvarial specimens were removed from the skull and placed onto a fixed plate with the dorsal aspect facing downward into an Instron 5565A load frame. A cancellous bone punch (tip radius = 0.15mm) was used for indenting the bone. It was attached to the upper load cell (50N

tension-compression capacity) which descended with the crosshead at a rate of 0.5mm per minute. Upon touching the basal surface of the calvaria, a 10-cycle compression-relaxation test was performed to account for hysteresis between 1.0 and 0.5N. Following this applied load, the stiffness modulus was determined by applying a compressive load to 4N. This resulted in an indentation of approximately 50μm depending on the modulus of the specimen. Curve fitting and modulus calculation were determined automatically with the load frame software (Bluehill from Instron). Six measures were taken from each specimen in the parietal section of the calvariae. These were taken on the left and right sides, including one from the center portion, a second from the lateral anterior edge and the last from the medial posterior edge. In most cases, an initial test indentation was performed to assure proper setup and fixation. The location of each test was recorded and matched with a corresponding microCT image taken previously. Thicknesses at each tested region were determined using image analysis software (ImageJ – from the National Institutes of Health) calibrated to the microCT images. Specimen strengths were calculated based indentation stiffness taking into account the thickness measures as determined by micro-CT, and the differences between the means of the two groups were compared using a standard Student-T test. Significance was set at $p < 0.05$. Results are presented as mean ± SD in text and Table 1. For graphical clarity, data are presented as mean ± SE in Figure 4.

This section, in part, is a reprint of the material as it appears in Bone. Zhang B, Cory E, Bhattacharya R, Sah R, Hargens AR (2013) Fifteen Days Microgravity Causes

Growth in Calvaria of Mice. The dissertation author was a co-investigator and third author of this paper.

Results

Changes in the weight and the parameters of bone adaptation to microgravity measured in the current study among the spaceflight and the ground control groups are summarized in Table 1. Mice from both the ground control and spaceflight groups were judged to be healthy by a veterinarian at NASA Kennedy Space Center. During the first two days, mice from the spaceflight group decreased their food intake, possibly attributed to shuttle takeoff and the initial experience of microgravity. Their appetite returned to normal after the two-day adjustment period. The ground control mice did not demonstrate any changes in food consumption during the experimental period. In the ground control group, each mouse lost on average 1.51 grams of weight from an average of 26.16 grams pre cageloading to 24.65 grams at cage-unloading, with an SD of 1.49. Each mouse from the spaceflight group lost on average 2.82 grams of weight from 26.20 grams before spaceflight to 23.38 grams upon mission completion, with an SD of 0.95. None of the mice in either group gained weight. The difference in weight changes between these two groups showed a trend of more aggressive weight loss in the spaceflight group compared to the ground control group ($p=0.07$). The weight loss observed in the spaceflight group may be attributed to several factors. Following transition to microgravity, the mice's appetite was temporarily reduced as they adapted to a new gravitational environment. Also, since load was removed from the appendages and majority of the body due to lack of gravity, both muscle atrophy and decreased bone density in the legs and spine likely occurred during the fifteen days of space travel. A third factor may be reduced circulating blood volume. These phenomena are well-documented in previous studies of post-flight

mice and in astronauts (Johnston et al., Gridley et al.). In fact, weight reduction in astronauts is mainly a result of fluid loss (60%), with fat utilization (30%) and muscle catabolism (10%) accounting for the remainder. Since neither food consumption nor physical activity were quantified during the experiment, it is difficult to attribute this weight loss to any particular reason. Furthermore, the SD of 1.49 grams demonstrates that there was great variability in the amount of weight lost by each mouse, which may reflect the variability of the individual animal.

Comparing murine calvariae, BV was increased ($p<0.05$) from 1.758 ± 0.122mm3(Mean ±SD) in the ground control group to 1.904 ± 0.084 mm3 in the spaceflight group (Fig. 3A). Correspondingly, Ct.Th showed a trend of an increase ($p=0.12$) from a mean of 0.099 ± 0.006 mm in the ground control group to 0.104 ± 0.005mm in the spaceflight group (Fig. 3B). There was no apparent effect on TMD ($p=0.31$), with a mean of 0.878 ± 0.029g/cc in the ground control group and 0.893 ± 0.028g/cc in the spaceflight group (Fig. 3C). Of the three parameters used to determine bone growth, calvaria BV from the spaceflight group exhibited a statistically significant increase of 8.3% (1.904/1.758 – 1) over the ground control group. Ct.Th indicated a trend of increase at 5.1% (0.104/0.099 - 1). Increase in TMD between the two groups was much less at 1.8% (0.893/0.878 – 1). Comparison between the groups shows that in addition to increased average thickness of the calvaria in microgravity, there probably was some bone expansion into air sinuses within the parietal bones (Fig. 2). The sagittal sutures of each calvaria was examined carefully, and the presence of bone fusion or bridging between the adjacent parietal bones was not observed.

Significant differences in the calvaria were reported from biomechanical analysis as well. The standard thickness that was manually set on the device was 400 microns. Calculated elastic modulus from the data program was thus divided by this thickness and multiplied by the measured CsTh by micro-CT in order to account for the variability in thicknesses between the calvaria. The average elastic modulus of the spaceflight calvaria taken over 6 points was 10.5 ± 1.9 GPa, significantly greater than that of the ground controls, 9.3 ± 2.1 GPa ($p<0.05$). This indicates that on average, the spaceflight calvaria were 12.9% stiffer than the ground controls, supporting the micro-CT data and our hypothesis of adaptive growth. Data compared between corresponding regions of the calvariae were not significant, likely due to lack of data points, with the exception of the left posterior region to the right of the suture within the volume of interest with an elastic modulus of 10.4 ± 1.5 GPa for the spaceflight group compared to 7.4 ± 2.1 GPa ($p<0.05$).

This section, in part, is a reprint of the material as it appears in Bone. Zhang B, Cory E, Bhattacharya R, Sah R, Hargens AR (2013) Fifteen Days Microgravity Causes Growth in Calvaria of Mice. The dissertation author was a co-investigator and third author of this paper.

Discussion

Bone loss in astronauts continues to be a major source of concern, and several studies have been conducted regarding the changes that occur in the human body secondary to microgravity exposure, including on cellular changes (Blaber, et al., 2013), the cardiovascular system (Arbeille, et al., 2001; Hughson, 2009), fluid shifts, and overall bone changes and loss (Baecker et al., 2003; LeBlanc et al., 2007). However, this is the first study, to our knowledge, to examine the adaptations of the calvarial bones in microgravity.

The purpose of this research was to determine whether or not the skull would adapt to the proposed head-ward fluid shift as other parts of the body have been shown to do. Due to restrictions that would occur in a study which examines the effects of microgravity on human bones, the mouse model was utilized and four parameters were established for analysis of bone: bone volume/total volume (BV/TV), cross-sectional thickness (CsTh), tissue mineral density (TMD), and elastic modulus.

To measure bone growth, calvariae were scanned by micro-CT, and the spaceflight group showed increased BV/TV compared to the ground controls. With the loss of head-to-foot gravity that is present on Earth, intracranial pressure increases through several mechanisms, namely the upward movement of cerebrospinal, venous and arterial fluids. Contrary to what has been recorded in head-down tail suspended rats, the mouse model exhibited decreased vasoconstriction and increased compliance in cerebral arteries, causing increased cerebral blood flow (Wilkerson et al., 2005; Taylor et al., 2013). As fluid shifts away from the lower extremities, it is absorbed into the cranial

tissues, causing facial puffiness, headaches, congestion, changes in taste and problems with vision (Mader, et al., 2011). This movement of excess fluid and subsequent eliminations, coupled with reduced use of load-bearing bones, leads to adaptation of the lower limbs as volume, mineral density and stiffness are reduced. In addition, as bone is lost from the load-bearing bones in the body, there appears to be some redistribution that occurs from the lower extremities to the head (Bikle, et al., 2003).

As noted by van Loon et al., (1996), skeletal unloading leads to a reduction in bone mineral density, sometimes referred to as mechanical inferiority. In individuals who engage in weight-lifting or strenuous exercise, bone mass may be increased; the bones adapt to the pressure put upon them. Research from Cosmos missions 1514, 1667 and 1887 determined that bone formation is diminished during spaceflight with results that support the hypothesis that bone loss or gain is responsive to the magnitude, direction, and frequency of stress placed upon them (van Loon et al., 1996). Osteocytes, mature osteoblasts that have become encased within the bone matrix, are believed to serve as a sensor of mechanical stimuli within bone tissue, not only through detection of mechanical load but also structurally as they adapt to the bone matrix to counter it. In a study by Taylor et al. (2007), researchers hypothesized that, as osteocytes can neither form nor resorb bone, they must contain the properties to "orchestrate mechanically induced bone remodelling" through the coordination of activities in the cells that reside on bone surface, such as osteoblasts. In their study, researchers built an osteocyte-osteoblast coculture model that was designed to mimic in vivo systems, providing for the researchers to expose the osteocytes to physiological fluid shear while, at the same time, separating osteoblasts from it. The results reflected that osteocytes exposed to a shear rate

of 4.4 dyn/cm^2 will increase the activity of the alkaline phosphatase in the protected osteoblasts, an indicator of active bone formation. The study further found that mitogen-activated protein kinase and functional gap junctional intercellular communication, along with extracellular signal-regulated kinase 1/2 signaling pathway, are essential to the response by osteoblasts to osteocyte mechanical signals. This action has been found to be unique function of osteocytes, and is not reproduced by any other mesenchymal cell types.

Bonewald & Johnson (2008) further note that mechanical loading suppresses production of sclerostin by osteocytes. Sclerostin inhibits the Wnt/B-catenin pathway that stimulates osteoblasts, supporting the notion that mechanical loading stimulates bone growth, which further corresponds with the findings of Robling et al. (2006), who found that mechanical stimulation in vivo reduces osteocyte expression of sclerostin, leading to ongoing bone growth. In their attempts to further examine the means by which bone cells are stimulated, Smalt et al. (1997) found that mechanical loading of bone may be sensed by osteoblastic cells through fluid flow-mediated wall-shear stress, as their studies showed that exposure of osteoblastic cells to increased fluid flow resulted in the production of nitric oxide and prostaglandin production. As these findings support the premise that fluid flow and loading the skull lead to elevated osteoblastic activity, it is likely that the increased intra-cranial pressure and cerebral blood flow that occurs in the upper body during spaceflight causes similar adaptation in the skull.

Hind-limb unloading studies in rats support findings of bone remodeling due to interstitial flow fluid (Bergula et al., 1999). These studies have shown to cause decreased perfusion of distal extremities as well as a corresponding rostral increase in fluid pressure

(Roer et al., 1990). Decreased bone mass in the femur and tibia, along with increased bone mass in the skull, mandible, clavicle and humerus correspond to changes in perfusion (Colleran et al., 2000), This study utilized hind-limb unloaded (HU) rats to examine the rates of perfusion in the forelimbs and head versus the hind limbs. Utilizing radiolabeled microspheres, skeletal perfusion was measured in the control standing group and at 10 minutes, seven days and twenty-eight days of HU. Data revealed reduced femoral and tibial perfusion within 10 minutes and diminished blood flow to the femoral shaft and marrow following 28 days of HU, as well as lowered mass of femora and tibiae after 28 days HU. In addition, their study found increased blood flow to the skull, mandible, clavicle and humerus after 10 minutes before returneing to control levels after 7 days, as well as increased mandibular (+10%, $P < 0.05$), clavicular (+18%, $P < 0.05$) and humeral (+8%, $P < 0.1$) mass with chronic HU; these results support the hypothesis that changes in bone blood flow act as a stimulus for bone remodeling during periods of microgravity (Colleran et al., 2000).

Other observations by Hillsley and Frangos (1994) associate bone remodeling with the changes in interstitial fluid flow and the mechanical unloading that occurs in a microgravity environment. The researchers hypothesized that increases in interstitial fluid flow and mechanical strain would result in remodeling of the skull, however the majority of research on bone remodeling in the upper body are mostly generated from bed rest and hind-limb unloading studies. Some extended bed-rest and lower body inactivity studies have resulted in findings that do pertain to skull remodeling. Studies by Beller et al. (2011) and Uebelhart et al. (2000) have demonstrated patterns of increased bone mineral

density in skull bone accompanied by mineral loss in the bones of the lower axial and appendicular skeleton. Whole-body composition was monitored by dual energy X-ray absorptiometry and bone and connective tissue metabolism was measured by biochemical markers and calcium regulating hormones on eight male volunteers during six weeks of anti-orthostatic bedrest followed by a reambulation period of one month. This study uncovered a trend of an increase in skull BMD that corresponded to a decrease in trunk, lumbar vertebrae and lower limb BMD (Uebelhart et al., 2000), corresponding with findings of the current study.

Following the micro-CT scanning of calvariae, micro-indentation testing was also carried out to examine changes in stiffening of the bone. According to Zhang et al. (2008), the stiffness or thickness of bone can be measured to determine stress and strain caused by loading at the cellular level by micro-indentation testing and is particularly useful in determining environmental effects on bone strength and mineral content. Results of these tests determined that the mean elastic modulus of the spaceflight group was significantly greater than that of the ground control group. As stiffness of the bone would correlate with bone growth, these results are consistent with the micro-CT data.

While these parameters show significant changes, the other two parameters, cross-sectional thickness and tissue mineral density showed similar adaptive trends, however not to a statistically significant level. This is likely due to the short duration (15 days) of microgravity exposure and the small sample size, denoting limitations in our study.

As prolonged spaceflight becomes feasible, it is increasingly important to determine the effects of long-duration spaceflight on the human body in order to maintain the health and safety of the astronauts on these long-distance missions. Research has

outlined many of the changes that occur during flights, and the focus is now on the development of effective countermeasures to mitigate these physiological consequences of microgravity. Techniques such as lower body negative pressure (LBNP) (Aratow, et al., 1993), artificial gravity (Caiozzo et al., 2009) and aerobic exercise (Trappe et al., 2009) are being explored to limit these health risks during and after the mission. In addition, there is recent attention on intracranial pressure and its effects on elevated intraocular pressure, causing vision impairments in cosmonauts. A recent study by Kramer at al. (2012) found optical abnormalities similar to those found in patients suffering from intracranial hypertension. Through magnetic resonance imaging (MRI) of the eyes and brains of 27 astronauts who spend an average of 108 days in spaceflight or on the International Space Station, various abnormalities were found, including flattening of the back of the eyeball, expansion of cerebrospinal fluid space surrounding the optic nerve, bulging of the optic nerve and changes within the pituitary gland and its connection to the brain. Ongoing studies in our lab are being conducted to determine whether intra-ocular pressure is increased following head-down tilt and whether LBNP is able to prevent these changes.

While this research studying the effects of microgravity on bone remodeling in the skull is the first of its kind, it is imperative to understand how to counter the fluid shift and develop a protocol to better ensure astronaut safety.

TABLE 1: Pre cage-loading and post cage-loading changes experienced by ground control and spaceflight groups in weight and in calvariae bone volume, average cortical thickness, tissue mineral density, and elastic modulus. Data are expressed as mean ± SD.

	Pre-Cage Loading (g)	Post-Cage Loading (g)	Change in weight (g)
Ground control mice	26.16 ± 1.29	24.65 ± 0.97	- 1.51 ± 1.49
Spaceflight mice	26.20 ± 0.76	23.38 ± 1.68	- 2.82 ± 0.95

	BV (mm3)	CsTh (mm)	TMD (g/cc)	Elastic Modulus (GPa)
Ground control mice	1.758 ± 0.122	0.099 ± 0.006	0.878 ± 0.029	9.3 ± 2.1
Spaceflight mice	1.904 ± 0.084	0.104 ± 0.005	0.893 ± 0.028	10.5 ± 1.9

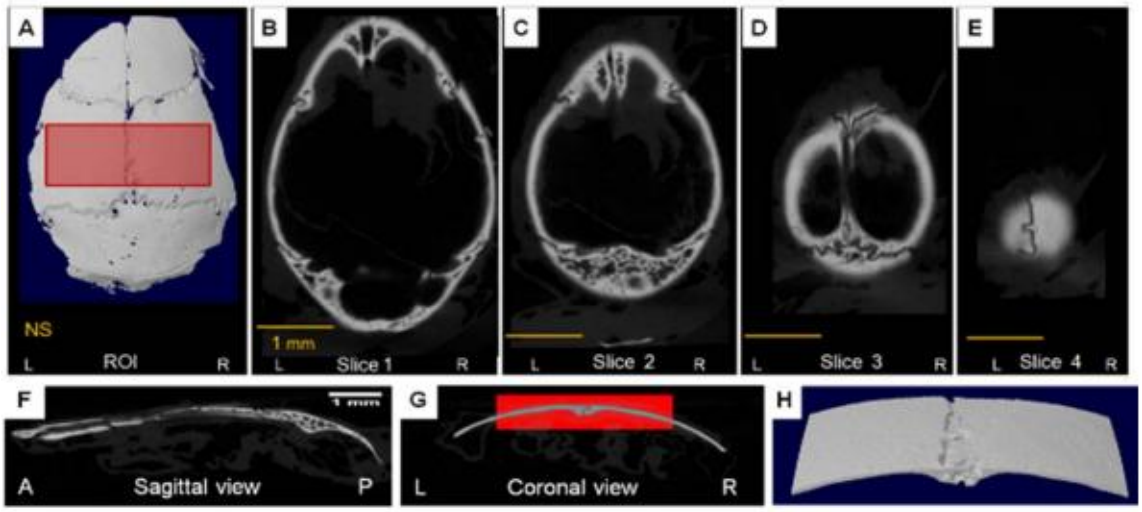

FIGURE 1: MicroCT scan of a murine calvariae from the spaceflight group presented in different views. Panel A: Calvaria with volume of interest (VOI), which is the rectangular volume placed on the parietal bones to determine calvaria growth. The parietal bones are located at the center of the murine calvaria, flanked by a pair of frontal bones rostrally and a singular interparietal bone caudally. Panels B – E: Transaxial slices taken 0.3 mm apart from the base to the top of the calvaria. Panel E captures the sagittal suture between the two parietal bones. Panel F: Sagittal view of the calvaria. Panel G: Coronal view of the calvaria with the volume of interest. Note the sagittal suture from the joining of the parietal bones. Panel H: Volume of interest is isolated and rotated 30º.

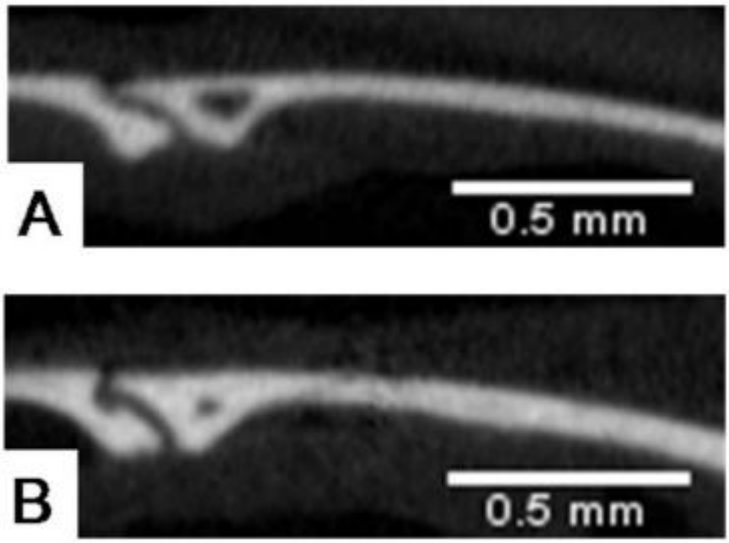

FIGURE 2: Calvaria from spaceflight group (panel B) showed increase in cross-sectional thickness compared to calvaria from ground control group (panel A). Bone also expanded into the cavity in the parietal bone adjacent to the suture. Note that these two images are purposely chosen to visualize contrast; comparison between calvariae randomly selected from ground control and spaceflight groups most likely would not demonstrate such overt changes.

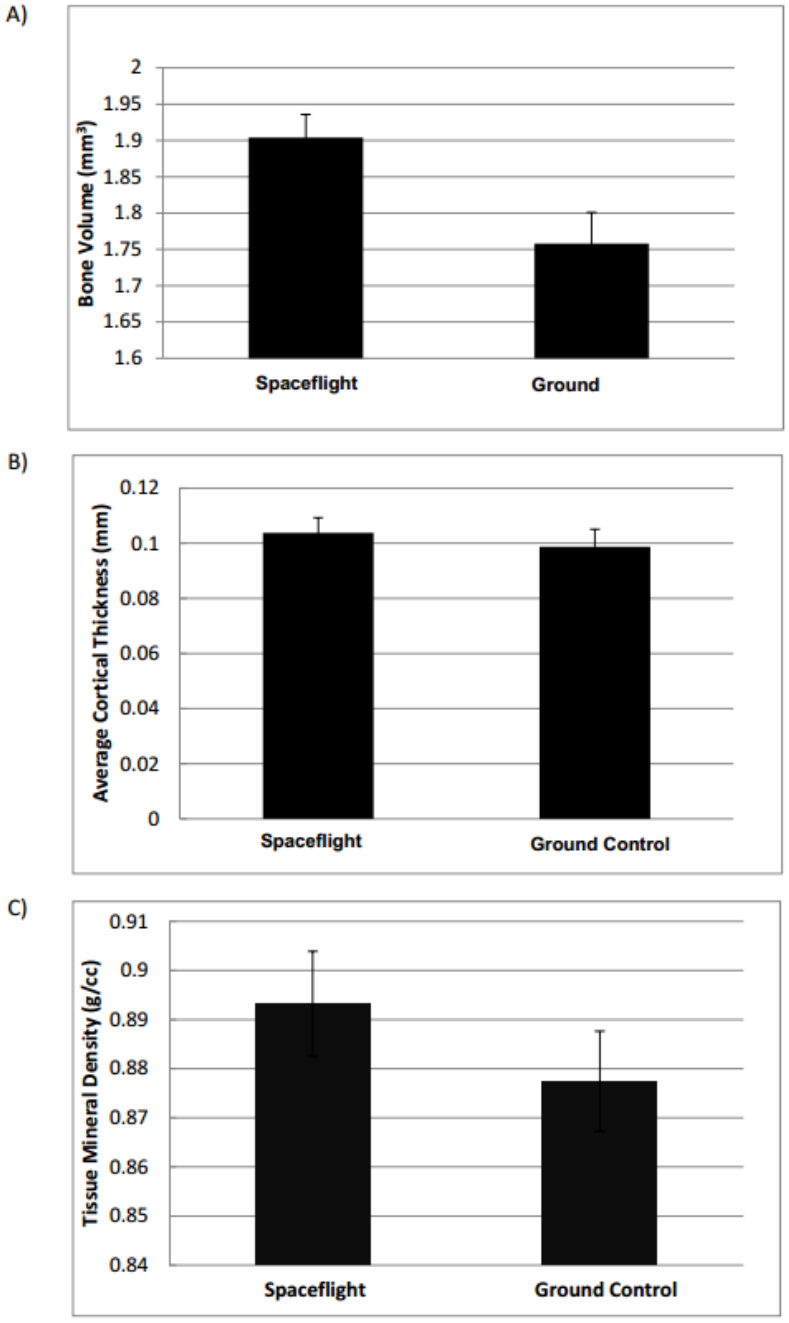

FIGURE 3: Comparison of ground control and spaceflight groups for bone volume, cross sectional bone thickness, and tissue mineral density. Data are mean ± SE; n = 7 in Spaceflight, 8 in Ground Control groups. Microgravity-induced bone remodeling was reflected by increases in all three parameters we used to define calvaria growth, but only the bone volume measurement reached significance between the groups.

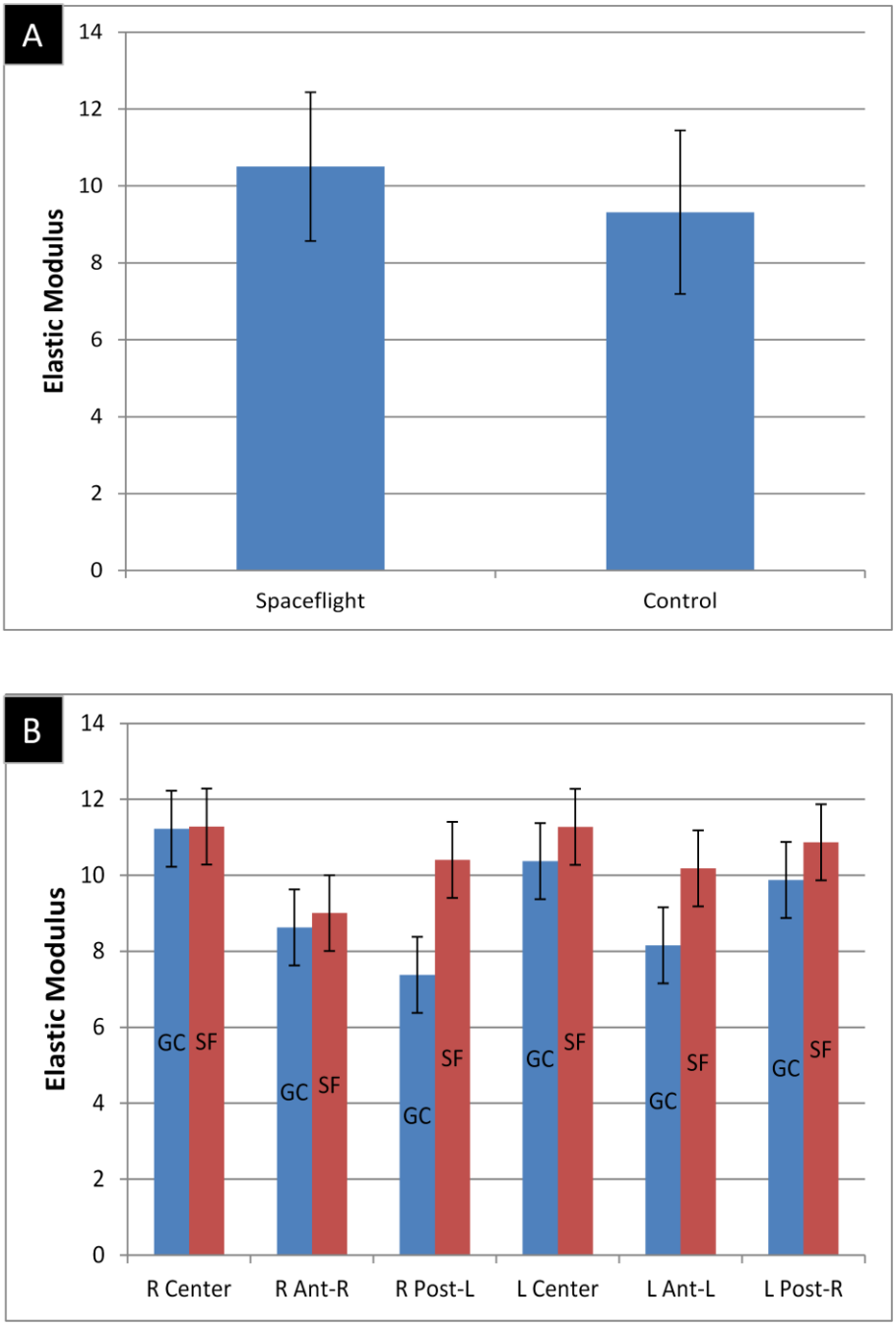

FIGURE 4: Comparison of ground control and spaceflight groups for elastic modulus. Data are mean ± SE; n = 7 in Spaceflight, 8 in Ground Control groups. The data over all regions as well as the R Post-L region alone was statistically greater in the spaceflight group compared to the control ($p<0.05$).

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