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Thirty days of spaceflight does not alter murine calvariae structure despite increased *Sost* expression☆

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ABSTRACT

Previously our laboratory documented increases in calvaria bone volume and thickness in mice exposed to 15 days of spaceflight aboard the NASA Shuttle mission STS-131. However, the tissues were not processed for gene expression studies to determine what bone formation pathways might contribute to these structural adaptations. Therefore, this study was designed to investigate both the structural and molecular changes in mice calvariae after a longer duration of spaceflight. The primary purpose was to determine the calvaria bone volume and thickness of mice exposed to 30 days of spaceflight using micro-computed tomography for comparison with our previous findings. Because sclerostin, the secreted glycoprotein of the *Sost* gene, is a potent inhibitor of bone formation, our second aim was to quantify *Sost* mRNA expression using quantitative PCR. Calvariae were obtained from six mice aboard the Russian 30-day Bion-M1 biosatellite and seven ground controls. In mice exposed to 30 days of spaceflight, calvaria bone structure was not significantly different from that of their controls (bone volume was about 5% lower in spaceflight mice, $p = 0.534$). However, *Sost* mRNA expression was 16-fold (16.4 ± 0.4, $p < 0.001$) greater in the spaceflight group than that in the ground control group. Therefore, bone formation may have been suppressed in mice exposed to 30 days of spaceflight. Genetic responsiveness (e.g. sex or strain of animals) or in-flight environmental conditions other than microgravity (e.g. pCO_2 levels) may have elicited different bone adaptations in STS-131 and Bion-M1 mice. Although structural results were not significant, this study provides biochemical evidence that calvaria mechanotransduction pathways may be altered during spaceflight, which could reflect vascular and interstitial fluid adaptations in non-weight bearing bones. Future studies are warranted to elucidate the processes that mediate these effects and the factors responsible for discordant calvaria bone adaptations between STS-131 and Bion-M1 mice.

1. Introduction

Preventing bone loss during long-duration spaceflight missions is of profound importance to mitigate the risk of bone fracture during and after flight (Smith et al., 2015). Although there is substantial inter-individual variation, the greatest decreases in bone mineral density are found in weight-bearing bones suggesting that reduced mechanical loading is a primary contributor to bone loss during spaceflight (LeBlanc et al., 2007; Vico et al., 2000). Recent advances in spaceflight exercise and nutritional countermeasures attenuate some bone loss at sites with a high-risk of fracture (Smith et al., 2012). However, while the primary goals for countermeasure efficacy are based on supplying mechanical loads and essential nutrients, bone capillary and interstitial fluid pressures may be additional factors affecting bone remodeling (Parazynski et al., 1991). Loss of hydrostatic pressure gradients in microgravity may be associated with the redistribution of bone mineral from the tibia to the skull in both human and rodent spaceflight studies (Zhang et al., 2013; Lafage-Proust et al., 1998; Oganov, 2006). These discoveries highlight the importance of understanding the multiple mechanisms by which microgravity exposure affects bone remodeling,

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for the development of optimal countermeasures.

The unloaded bones of the skull present a unique opportunity to investigate the effects of fluid dynamics or other unknown factors on bone remodeling, independent of musculoskeletal unloading. Previous studies have documented augmented skull bone mass after simulated spaceflight. For example, skull weight and bone mineral density are increased in hindlimb unloaded rats (Colleran et al., 2000; Roer and Dillaman, 1990; Navidi et al., 1995; Arnaud et al., 1994), suggesting that factors associated with this microgravity analog, such as headward fluid shifts, are sufficient for causing alterations in skull-bone remodeling. These results are consistent with human bed rest studies. A trend toward increased skull bone mineral density was observed after 6 weeks of bed rest, and significant increases were observed after 17 weeks of bed rest (Leblanc et al., 1990; Uebelhart et al., 2000). However, it is unclear whether the observed adaptations of unloaded bones to simulated spaceflight reflect the remodeling of these bones in actual microgravity environments.

Skull bone adaptations have also been reported in astronauts after actual microgravity exposure (Oganov, 2006; Miyamoto et al., 1998). For example, bone mineral density increased (2.8 ± 2.2%) in the heads of Mir 6–9 crewmembers (n = 7) after flights lasting 132–176 days (Oganov, 2006). However, data on adaptations of unloaded bones after spaceflight are scarce and most of our understanding has been generated from murine studies. For example, calcium and phosphorus concentrations increase in parietal bones of rats exposed to 14 days of spaceflight, relative to ground controls (Lafage-Proust et al., 1998). More recently, mice exposed to 15 days of microgravity aboard the National Aeronautics and Space Administration (NASA) shuttle mission STS-131 had greater calvarial bone volume (~8.3%) and calvarial cortical thickness (~5.1%) than their ground controls (Zhang et al., 2013). Further studies are necessary to characterize these adaptations and elucidate the biochemical pathways of remodeling that are altered during spaceflight. Understanding the mechanisms that lead to calvarial bone augmentation in microgravity conditions has important implications for the development of countermeasures to adverse bone adaptation during spaceflight. Moreover, additional data are required to define the contribution of headward fluid shifts to bone homeostasis.

Sclerostin, the secreted glycoprotein of the *Sost* gene, is a potent inhibitor of bone formation and is specifically expressed by mature osteocytes (Poole et al., 2005). Because osteocytes are the putative primary cells for sensing mechanical loading in bone (Robling et al., 2006), sclerostin may play a key role in the mechanotransduction pathway of altered fluid pressures (Bloomfield, 2006). In a previous study examining rat tibiae during hindlimb unloading, increased sclerostin expression was associated with suppressed cortical bone mineral growth and periosteal bone formation rate (Macias et al., 2012). However, the extent to which mechanical unloading and altered fluid pressures independently affect sclerostin expression and bone adaptation in vivo is unclear, making the unloaded bones of the skull a key target of study.

The purpose of this study was twofold. Our first aim was to assess the structural properties of calvaria from mice flown on the 30-day Bion-M1 biosatellite using micro-computed tomography analysis for comparison to our previously-published data on calvaria from mice flown on the 15-day NASA shuttle mission STS-131 (Zhang et al., 2013). Our second aim was to quantify *Sost* gene mRNA expression using standard quantitative PCR (qPCR) techniques. We hypothesized that 30 days of spaceflight increases bone volume and cortical thickness in mice calvariae with corresponding reductions in *Sost* mRNA expression.

2. Methods

2.1. Ethical statement

The study on Bion-M1 mice was approved by the Biomedical Ethics Committee of the Russian Federation State Research Center Institute for Biomedical Problems (IBMP), and the Institutional and Animal Care and Use Committees (IACUC) of Moscow State University's (MSU) Institute of Mitoengineering and NASA. Experimental protocols were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. All Bion-M1 mice were purchased healthy from the Animal Breeding Facility - Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry.

2.2. Bion-M1

Experiments were performed with 13 male, specific pathogen-free, C57BL/6N mice. All mice were between 19 and 20 weeks old at the start of experiments. Six mice, representing the spaceflight group (SF, n = 6), experienced 30 days of microgravity aboard the 30-day Bion-M1 biosatellite. Seven mice, representing the ground control group (GC, n = 7), were maintained on land under normal gravitational loads. Both groups were housed in BOS (Block Obespecheniya Soderzhaniya) habitats, with three mice in each BOS. Spaceflight mice were loaded onto the unmanned Bion-M1 biosatellite that was launched on April 19, 2013, and returned on May 19, 2013. The BOS habitats were cleaned and refurbished after flight, and loaded with ground control mice from July 26 to August 26, 2016. Both groups were housed on a 12-hour light-dark cycle and consumed a paste food diet containing 74.6% H2O developed by Russia's IBMP. Environmental conditions were controlled to be identical, including temperature, humidity, gas composition (pCO_2 of about 0.1 mm Hg) and other spaceflight-specific parameters. Gravity, or lack thereof, was therefore the primary environmental difference between the groups. Further information on housing conditions, environmental parameters, diet, and training for these animals has been described in detail previously (Andreev-Andrievskiy et al., 2014).

The descent module landed in the Orenburg region of Russia, where recovery personnel retrieved the mice and performed initial health inspections onsite in a field laboratory. The spaceflight mice were then transported to the IBMP in Moscow, Russia and euthanized 13–15 h after landing by cervical dislocation. To isolate parietal bones, samples were cut caudal to the coronal suture and caudal to the anterior lamboid suture. They were then frozen in liquid nitrogen and shipped under dry ice to our laboratory at UCSD for analysis.

2.3. Micro-computed tomography

Imaging, analyses, and parameter calculations were similar to those described in detail by Zhang and colleagues (Zhang et al., 2013). Samples were imaged on a micro-computed tomography scanner, Skyscan 1076 (Bruker microCT, Kontich, Belgium), in accordance with established guidelines (Bouxsein et al., 2010). Calvariae were wrapped in tissue paper moistened with phosphate buffered saline (PBS) and scanned using a 9 μm voxel size, an electrical potential of 50 kVp and current of 200 μA, and a 0.5 mm aluminum filter. Images were calibrated against 2-mm diameter hydroxyapatite rods (250 and 750 mg/cm^3) to determine tissue mineral density (TMD) with a beam hardening correction algorithm applied during image reconstruction.

Bone structure and histomorphometric parameters were visualized and determined using SkyScan CT-analyser and DataViewer software (Kontich, Belgium). A standardized rectangular volume of interest (VOI, 18.1 mm^3, 5.5 mm × 1.2 mm area in the coronal plane, 2.7 mm depth) was located at the center of the parietal bones. Three different thresholding modes were applied separately to segment out the bone: a global threshold and two adaptive thresholding algorithms (with either 8- or 10-pixel radius). The global algorithm, the same method applied in the 15-day microgravity study, is ideal for binarizing thick non-porous structures, such as cortical bone. However, tissues displayed high cortical porosity consisting of a network of canals which provide conduits for vasculature. Therefore, the adaptive algorithms were used to

provide an accurate binarization of thin or network-like structures; for each pixel, the intensities of the surrounding pixels specified in a given radius are taken into account for segmentation.

After the images were binarized, an erosion of one pixel was performed to eliminate partial-volume effects and calculate the following parameters: total volume (TV; set to 18.111 mm^3 for each sample), bone volume (BV; defined as the volume within the VOI occupied by bone), bone volume fraction (BV/TV), average cortical thickness (measured as the average 2D thickness of the calvariae bone in the coronal plane), and TMD. Cortical thickness was calculated by dividing the BV (mm^3) by the area of the sub-pericranial surface (mm^2) within the VOI. TMD was calculated by dividing the bone mineral content (g) obtained from calibration with hydroxyapatite rods by the BV (cm^3).

Previous guidelines for characterizing bone morphology with micro-CT are intended for reporting values derived from a cylindrical VOI containing both cortical and trabecular bones (e.g. the femur) (Bouxsein et al., 2010). As described by Zhang and colleagues (Zhang et al., 2013), this standard for a minimal set of reportable variables does not fully apply to our samples because calvariae are solely composed of cortical bone. For example, the total cross-sectional area inside the periosteal envelope is not applicable to calvariae, and would be equivalent to the cortical bone area (cortical volume / (number of slices ∗ slice thickness)). We chose to present the BV (equivalent to cortical volume) instead of cortical bone area because BV is a more accurate representation of the differences between the spaceflight and ground control groups. In addition, not all image slices within the VOI contain calvarial bone, unlike that expected when the VOI is localized length-wise to the mid-section of a long bone.

2.4. Quantitative polymerase chain reaction (qPCR)

The pre-frontal regions of six calvariae from each group were used for qPCR analyses. The tissue samples were powdered in a homogenizer and suspended in a TRIzol (Invitrogen) solution for 30 min to completely disassociate the nucleoprotein complex. To facilitate phase separation, 200 µl of chloroform was added, and the solution was shaken by hand and centrifuged at 12,000 rpm for 15 min. The supernatant was transferred to an Eppendorf tube containing an equal volume of 70% ethanol. After total RNA was precipitated, the solution was transferred to a RNA binding column, and RNA purification was carried out according to the manufacturer's instructions (Purelink RNA Mini Kit, Life Technologies, Carlsbad, CA). Pure RNA was eluted from the columns in 30 µl of RNase-free water. RNA concentration and purity was estimated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Normalized RNA (1 µg/µl) was converted to cDNA using a Qiagen kit with gDNA wipeout buffer. Reverse transcription was carried out at 42 °C for 30 min according to the manufacturer's instructions. The cDNA was stored at − 20 °C or used immediately. Gene expression analysis was performed using SYBR green (QuantiTect SYBR Green PCR, Qiagen, Germantown, MD) in a 96-well format on an Eppendorf Mastercycler machine. Primers were ordered from IDT Technologies with the following sequences for *Sost* qPCR: Sense-TCCTGAGAACAACCAGACCA and Antisense-GCAGCTGTACT CGGACACATC. This primer pair has previously been validated (Brunkow et al., 2001). The annealing temperature was standardized at 57 °C for 15 s. Three cycle threshold (CT) values were averaged for each animal. The results were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the standard, producing one ΔCT value for each animal. The Livak method was used to calculate the difference in expression between spaceflight and ground control mice (Livak and Schmittgen, 2001).

2.5. Statistics

Data were analyzed using nonparametric statistics because sample sizes were small for tests of normality (SF: n = 6; GC: n = 7) and visual inspection suggested that distributions were better represented by the median. Independent-samples Mann-Whitney *U* Tests (IBM SPSS Statistics Version 21) were used for hypothesis testing to determine significant differences between the ground control and spaceflight groups. The alpha level for statistical significance was set to $p < 0.05$. Results are presented as median ± interquartile range. Comparisons between groups are presented as a percent difference in medians.

Table 1
Structural properties of 30-day mouse calvariae from micro-computed tomography analyses: bone volume fraction (BV/TV), cortical thickness, and tissue mineral density (TMD). Three different algorithms were used for each sample: global, 8-pixel, and 10-pixel. Spaceflight (SF) and ground control (GC) groups were compared using Mann-Whitney *U* tests. No significant structural differences were detected between the calvariae of mice exposed to 30 days of spaceflight and their controls ($p > 0.05$). Data are presented as median ± interquartile range.

	BV/TV (%)	Cortical thickness (mm)	TMD (g/cm^3)
Global			
GC (n = 7)	10.01 ± 0.91	0.100 ± 0.010	0.794 ± 0.010
SF (n = 6)	9.51 ± 1.26	0.103 ± 0.013	0.798 ± 0.055
8-Pixel			
GC (n = 7)	9.35 ± 0.73	0.067 ± 0.016	0.831 ± 0.024
SF (n = 6)	9.38 ± 1.04	0.072 ± 0.006	0.830 ± 0.070
10-Pixel			
GC (n = 7)	10.07 ± 0.92	0.079 ± 0.015	0.825 ± 0.026
SF (n = 6)	9.77 ± 1.27	0.078 ± 0.005	0.824 ± 0.071

3. Results

Previously our laboratory documented greater calvaria bone volume and thickness following 15 days of spaceflight, relative to ground controls (Zhang et al., 2013). However, when 30-day samples were analyzed using the same global thresholding algorithm as in our previous study, no significant structural differences were detected between the calvaria of mice exposed to 30 days of spaceflight and their controls (Table 1). Compared to the GC group, the SF group had about a 5.0% lower BV/TV percentage ($p = 0.534$), a 3.0% higher average cortical thickness ($p = 1.00$), and a 0.5% higher TMD ($p = 0.628$). However, increased cortical porosity was observed during image pre-processing in the 30-day samples compared to previous 15-day samples (Fig. 1). Therefore, it was appropriate to apply an adaptive algorithm for adequate segmentation of the bone structure. With either adaptive algorithm (8-pixel radius or 10-pixel radius), there were still no significant structural differences between the groups. For example, with an adaptive 10-pixel radius algorithm, the SF group had about a 3.0% lower BV/TV percentage ($p = 0.534$), a 1.3% lower cortical thickness ($p = 1.00$), and a 0.1% lower TMD ($p = 0.945$) than the GC group.

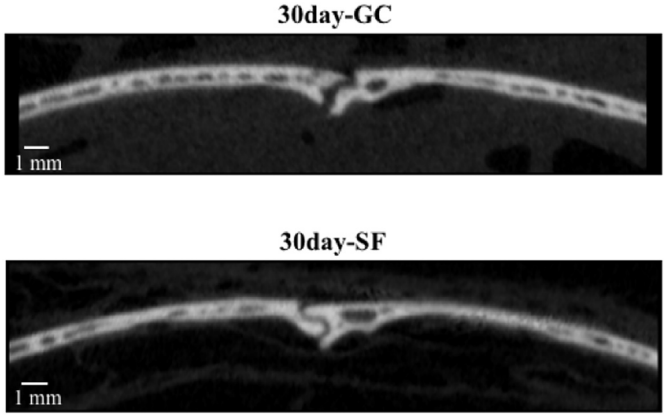

Fig. 1. Representative micro-computed tomography greyscale images of calvaria from mice exposed to 30 days of spaceflight (SF) and their ground controls (GC).

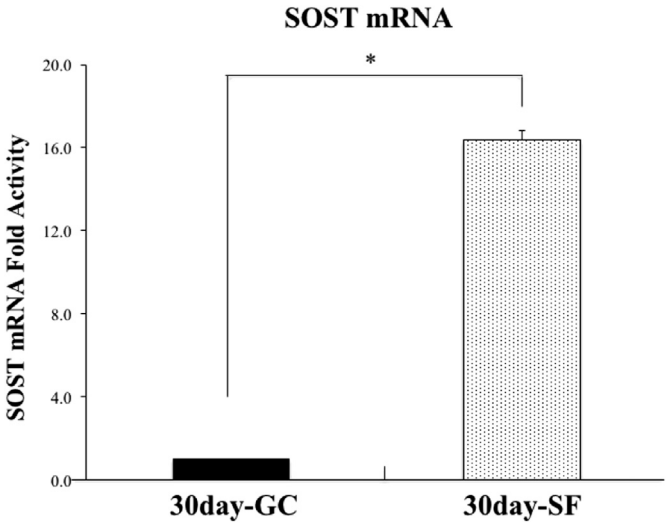

Fig. 2. Sost mRNA fold activity (using GAPDH as the standard) in mouse calvariae after 30 days of spaceflight. GC = ground control. SF = spaceflight. *Significant difference between spaceflight and ground control, p = 0.002.

Despite there being no significant differences in structural properties, a statistically significant difference was detected between groups in *Sost* mRNA expression. Nonparametric statistics were performed on ΔCT values according to Yuan and colleagues (Yuan et al., 2006). The median ΔCT value of the SF group (17.25 ± 1.78) was significantly lower than that of the GC group (21.61 ± 0.8, p = 0.002). Using the Livak method ($2^{-\Delta\Delta CT}$, mean ± SEM) (Livak and Schmittgen, 2001), the SF group had a 16-fold (16.4 ± 0.4, Fig. 2) greater mRNA expression than the GC group.

4. Discussion

Our previous study on mice exposed to 15 days of spaceflight indicated that microgravity causes responsive changes in calvarial bones that do not normally bear weight (Zhang et al., 2013). Because of the relatively short flight duration in our previous study, we hypothesized that a mission of longer duration would strengthen the magnitude of structural differences and better represent the impact of microgravity on the unloaded bones of the skull. However, the current 30-day spaceflight data do not support our hypothesis that spaceflight produces higher calvaria bone volume and cortical thickness. Rather, non-significant decreases in bone volume were observed in spaceflight animals, with no apparent changes in cortical thickness or tissue mineral density. Nonetheless, 30-day spaceflight samples demonstrate greater *Sost* mRNA gene expression.

Our results from Bion-M1 mice were surprising; however, this is not the first time that discordant results have been found between STS and Bion mice. For example, studies on cerebral basilar arteries revealed greater vascular distensibility and vasodilation in STS-135 mice compared to their ground controls, but virtually absent distensibility differences between Bion-M1 mice and their ground controls (Taylor et al., 2013; Sofronova et al., 2015). Although these studies collectively demonstrate that spaceflight impairs cerebral artery myogenic vasoconstriction, the differences in distensibility, along with the discordant results in our study, suggest that the age, sex, or strain of the animals studied, mission duration, time of euthanization, or environmental factors other than microgravity may modulate alterations in cerebral artery mechanical properties and skull bone remodeling during spaceflight.

A discussion examining the differences between STS-131 and Bion-M1 may provide insight into the factors that influence calvariae bone adaptations. Animal characteristics for Bion-M1 mice are described above and those for STS-131 mice have been described previously (Zhang et al., 2013). Previous evidence suggests that susceptibility to bone loss during hindlimb unloading is a genetically determined trait (Amblard et al., 2003). However, these comparisons were made between the femurs of male C3H/HeJ and male C57BL/6J mice (Amblard et al., 2003), whereas STS-131 mice were 23-week-old female C57BL/6 and Bion-M1 mice were 19–20-week-old male C57BL/6N. Therefore, it is unknown which of the differing characteristics, age, sex, or strain, may have contributed to bone adaptations after flight. In addition, within our current theoretical framework it is unlikely that the longer mission duration of our current study (30 days) would attenuate or even reverse the structural adaptations observed previously. In fact, evidence for murine skull bone adaptation after hindlimb unloading is typically found with durations similar to or longer than that of the current study (LeBlanc et al., 2007; Roer and Dillaman, 1990; Navidi et al., 1995; Arnaud et al., 1994). And even longer durations are required for adaptations to be observed in humans (Oganov, 2006; Leblanc et al., 1990; Uebelhart et al., 2000; Miyamoto et al., 1998).

Further differences between the mice of these studies were observed immediately upon landing. Bion-M1 habitats were designed to support mice without any human interference. While the nutritional status of mice post-flight was variable, an average weight increase was observed in the SF group (8%) and in the GC group (4%) (Andreev-Andrievskiy et al., 2014). In contrast, both the SF and GC groups of the 15-day STS-131 study lost weight (− 2.8% and − 1.5%, respectively) (Zhang et al., 2013). Based on previous research, moderate weight gain would not be expected to influence bone mass in skeletally mature animals (Turner and Iwaniec, 2010). However, these studies examined weight-bearing bones after a calorie-increased diet, and do not provide data on the unloaded bones of the skull. While spaceflight-induced weight change is thought to be due to decreased physical activity, this has not been determined in mice. Another difference is the amount of time for re-adaptation to Earth's gravity. Bion-M1 mice were euthanized 13–15 h after landing, whereas STS-131 mice were euthanized within 3–4 h after landing. Although bone mineral content would not be expected to change over this period, we cannot rule out the possibility that this difference may affect gene expression levels. In addition, calvariae from three mice were excluded from our STS-131 analysis because they were euthanized 28 h after landing, which raised concern that they may not truly reflect microgravity-induced changes and may instead introduce bias (Zhang et al., 2013). While these differences are important to consider, it is unclear how stress, weight change, or re-adaptation to gravity affect bone remodeling.

Flight conditions may be primary modifiable factors that moderate the effects of spaceflight on bone remodeling. Levels of in-flight pCO_2 are a potential cause for the differences in bone structure findings between the Space Shuttle docked to the ISS and the Bion biosatellite. Relative to Earth at sea level (~0.23 mm Hg), pCO_2 on the ISS was about 10 times higher (~2.5 mm Hg), while that on the Bion biosatellite was about 10 times lower (~0.01 mm Hg) (Andreev-Andrievskiy et al., 2014; Law et al., 2014). According to studies conducted on Earth, these relatively large changes may have effects on cerebral vascular tone. High CO_2 levels are known to lower extracellular pH, which induces cerebral vasodilation mediated by nitric oxide, cyclic nucleotides, prostanoids, potassium channels, and calcium ion exchange (Brian, 1998). As a result, cerebral blood flow is estimated to increase by 1–2 ml/100 g/min for each 1 mm Hg rise in arterial partial pressure of CO_2 (Iadecola and Zhang, 1996), and may cause an elevation of intracranial pressure. Although cerebral blood flow and cerebral blood volume change similarly during high CO_2 levels on Earth (Ito et al., 2005), this relationship may differ with microgravity conditions during spaceflight because of impaired venous drainage caused by headward fluid shifts, leading to greater increases in cerebral blood volume. It is possible that higher-than-normal CO_2 levels on STS-131 may have increased cerebral hemodynamics, which may have led to decreased *Sost* expression and augmented calvarial volume, whereas lower-than-normal CO_2 levels on Bion-M1 may have had the opposite effect.

However, further research is necessary to determine the tissue CO_2 levels during spaceflight and their relationship to bone adaptations and *Sost* expression in the skull. Other environmental factors include potentially confounding variables such as diet and galactic cosmic radiation.

Sclerostin is expressed by osteocytes and plays an important role in the mechanotransduction pathway of loading stimuli to bone (Poole et al., 2005; Lanyon, 1993). Sclerostin inhibits bone formation by binding with high affinity to Lrp5/6 and thereby suppressing Wnt signaling (Robling et al., 2006). In the absence of sclerostin, such as in *Sost* knockout mice (Li et al., 2008), LRP5/6 and Wnt/β-catenin signaling increase osteoblast proliferation and thus bone mass accrual (Williams and Insogna, 2009). Therefore, we hypothesize that decreased sclerostin expression leads to the previously observed augmented skull bone mass after spaceflight and hindlimb unloading (Zhang et al., 2013; Roer and Dillaman, 1990; Arnaud et al., 1994; Miyamoto et al., 1998). However, in the current study, we observed 16-fold greater *Sost* mRNA expression in flight calvariae, which would predict an increase in sclerostin protein expression and a decrease in bone formation. This 16-fold difference in Sost mRNA expression is large, and may partially reflect overall skeletal unloading because sclerostin circulates in the blood (Mödder et al., 2011), or re-adaptation to Earth's gravity. Unfortunately, technical issues prevented our inclusion of sclersotin protein expression in this study. In addition, although our structural results did not reach significance, the direction of change (a decrease in flight calvaria bone volume) would be coherent with the increased *Sost* mRNA expression. Future studies that investigate the full Wnt pathway may help explain the observed changes in bone volume.

To our knowledge, this is the first study to measure *Sost* expression levels in mouse calvariae after actual microgravity exposure. Numerous studies demonstrate that mechanical loading/unloading influences *Sost*/sclerostin expression, but few have examined the causes of remodeling in bones that are not mechanically loaded. For example, forearm loading in rats decreases *Sost* mRNA expression in the ulna, while hindlimb unloading increases *Sost* expression in the tibia (Robling et al., 2008). Similarly, the proportion of sclerostin-positive osteocytes is increased in the tibia of hindlimb unloaded rats, relative to controls (Macias et al., 2012). Furthermore, *Sost* knockout mice are resistant to mechanical unloading-induced bone loss and do not exhibit the characteristic decreases in Wnt/β-catenin activity with unloading (Lin et al., 2009). However, in unloaded bones, such as the skull, these biochemical responses must be influenced by alternative mechanisms. For example, during hindlimb unloading, skull bone perfusion changes correspond to the induced osteogenic responses (Colleran et al., 2000). These results suggest that altered bone blood flow can provide a stimulus for bone remodeling during spaceflight (Bloomfield, 2006). Combined with our biochemical data, they indicate that this response likely acts through modulation of *Sost*/sclerostin expression. In humans, the absence of hydrostatic pressure gradients causes fluids to shift toward the head (Hargens and Richardson, 2009), and likely increases cerebral blood flow and intracranial pressure (Mader et al., 2011; Draeger et al., 1994; Kramer et al., 2012). Unfortunately, to date, bone perfusion has not been measured in an actual microgravity environment.

Osteocytes may respond to interstitial fluid flow and shear stress within bone canaliculi induced by altered fluid dynamics or mechanical strain (Hillsley and Frangos, 1994). For example, loading-induced increases in bone formation rate are associated with voltage gradients (stress-generated potentials) that are generated by interstitial fluid flow (Turner et al., 1994). By an alternative mechanism, fluid shear stress may directly stimulate osteoblasts (Reich et al., 1990). In an animal model study, oscillatory interstitial fluid flow was induced in the absence of bone mechanical strain (Qin et al., 2003). While disuse resulted in significant bone loss, disuse plus 10 min per day of a sinusoidal fluid pressure stimulus increased bone mass in a non-uniform spatial distribution that was consistent with the induced fluid pressure gradient through the cortex. These results indicate that new bone formation is strongly correlated with fluid pressure gradients (Qin et al., 2003). Therefore, in the unloaded bones of the skull, altered bone remodeling is likely a response to microgravity-induced alterations in fluid pressures and/or fluid dynamics, and is probably mediated by osteocyte-modulated *Sost*/sclerostin expression levels. The effects of spaceflight on *Sost* expression observed in this study may be due to atypical intracranial pressures, altered skull interstitial or vascular fluid flow dynamics, a combination of the two, or other unknown environmental factors associated with spaceflight. But given their potential implications for bone adaptation and other physiological changes during spaceflight, future studies examining these mechanisms are warranted.

4.1. Limitations

We did not measure calvaria mechanical strain during spaceflight or on the ground. However, biochemical data pertaining to the calvarial mechanotransduction pathway data support the possibility that flight bone tissue was exposed to a mechanical loading environment different from that of controls. Unfortunately, we did not measure *Sost* mRNA levels in the 15-day mouse calvariae because the study was not designed to measure gene expression. Because this study was one of several investigations that together examined many different physiological systems, measures of dynamic histomorphometry were not permitted. Logistical requirements and concern that calcein administration would have unknown interactions with other systems affecting other planned investigations precluded this technique. Lastly, micro-computed tomography analyses show that 30-day tissues were more porous than the 15-day tissues, making it necessary to apply an adaptive algorithm for adequate segmentation of the bone structure. This cortical porosity was observed during image preprocessing for both the flight and the control samples. Therefore, it is unlikely that microgravity caused bone porosity. However, it is possible that other animal characteristics, such as mouse strain, sex, and age, could be related to these observed differences.

4.2. Conclusions

In contrast to previous findings with shorter flight duration, this study found no significant structural differences between the non-weight-bearing calvaria bones of mice exposed to 30 days of spaceflight and their ground controls. However, this study does provide biochemical evidence that calvaria mechanotransduction pathways are altered during spaceflight, which could reflect vascular and interstitial fluid adaptations to microgravity environments. Future studies elucidating the processes that mediate these effects and the factors responsible for discordant bone adaptations may lead to the development of countermeasures against bone loss caused by spaceflight conditions, bed rest, and osteoporosis.

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Authors' roles

Study design: BRM and ARH. Study Conduct: BRM, TRM, and JS. Data collection: BRM, TRM, and JS. Data analysis: BRM, TRM, and JS. Data interpretation: BRM, TRM, ARH, and JS. Drafting manuscript: TRM. Revising manuscript content: BRM, ARH, and JS. Approving final version of manuscript: BRM, TRM, ARH and JS. TRM takes responsibility for the integrity of the data analysis.

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