Fifteen Days Microgravity Causes Growth in Calvaria of Mice

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Abstract

Bone remodeling may occur in spaceflight as a response to skeletal unloading and head-ward fluid shifts. While unloading causes significant loss of bone mass and density in legs of animals exposed to microgravity, increased blood and interstitial fluid flows accompanying microgravity-induced fluid redistribution may elicit an opposite effect in the head. Seven C57BL/6 mice were randomly chosen for exposure to 15 days of microgravity on the STS-131 mission, while eight littermates served as ground controls. Upon mission completion, all 15 mice calvariae were imaged on a micro-computed tomography scanner. A standardized rectangular volume was placed on the parietal bones of each calvaria for analyses, and three parameters were determined to measure increased parietal bone volume: bone volume (BV), cross-sectional thickness (CsTh), and tissue mineral density (TMD). Microgravity exposure caused a statistically significant increase in BV of the space flight (SF) group compared to the ground control (GC) group – the mean $\pm$ SD for SF group was 10.48 ± 0.47 %, compared to 9.64 ± 0.79 % for GC group (p<0.05). CsTh demonstrated a trend of increase from 0.099 ± 0.006 mm in the GC group to 0.104 ± 0.005 mm in the SF group (p=0.12). TMD was similar between the two groups with 0.878 ± 0.029 g/cc for GC

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and 0.893 ± 0.028 g/cc for SF (p=0.31). Our results indicate that microgravity causes adaptive changes in calvarial bones that do not normally bear weight. These findings suggest that fluid shifts alone accompanying microgravity may initiate bone remodeling independent of skeletal loading by tissue.

Keywords

bone; adaptation; space; microgravity

Introduction

Skeletal adaptation to microgravity is a physiological obstacle that may limit prolonged space exploration [1]. Studies from astronauts demonstrate variable bone loss corresponding to mission length [2, 3, 4]. Weight-bearing bones such as the femur, calcaneus, hip, spine and neck lose more bone than non-weight-bearing bones [5], establishing the pivotal role of skeletal unloading on bone remodeling. Physiological studies of the Skylab missions document increasing calcium and phosphate levels in both serum and urine of crewmembers [6], reflecting a gradual loss of bone mineral content in these astronauts. Some of these crewmembers continue to lose bone after returning to Earth, and those who experience the greatest bone decrements do not fully recover even after five years [7]. The correlation between bone loss and diminishing bone strength suggests a significant risk of fractures for astronauts [8, 9, 10].

Development of countermeasures against the osteopenic effect of space travel requires a better understanding of bone adaptation. Human bed-rest is a method used to simulate microgravity without spaceflight. Using this technique, LeBlanc and team demonstrate the greatest bone loss in weight-bearing bones of the lower body, notably the lumbar spine, femur, tibia and the calcaneus [11]. A 60-day bed-rest study reports the greatest loss at the proximal femur and distal tibia [12]. These results are consistent with bone changes induced by spaceflight. Bone measurements on Skylab astronauts show increasing mineral loss in the os calcis corresponding to the length of the mission [4]. A more comprehensive comparison between 18 cosmonauts and bed-rest subjects supports that spaceflight produces similar, if not greater, mineral loss in weight-bearing bones than bed-rest [5]. Bones of the upper limbs are minimally impacted, and increases in bone mineral density (BMD) of the skull are observed [4, 11, 13].

To date, musculoskeletal research on physiological adaptations to microgravity focuses on weight-bearing regions of the lower body. While modest effort is made to study remodeling of partially-loaded bones (ie: the upper appendages) in response to weightlessness, data on non-loaded bones are scarce and are generated mostly from bed-rest and rodent hind-limb unloading studies. Studies of extended bed-rest and lower-body disuse demonstrate patterns of increased BMD in skull bones along with mineral loss in bones of the lower axial and appendicular skeleton [11, 13, 14, 15]. In their simulated weightlessness experiment, Roer and Dillaman found heavier dry and ash weights of the skulls from hindlimb-suspended rats [16]. However, it is unclear whether the observed adaptations of unloaded bones to

inactivity and simulated microgravity reflect the remodeling of these bones in actual microgravity.

The present paper attempts to quantify the adaptations of cranial bones to spaceflight. Specifically, our goals are to evaluate the remodeling of skull bones due to actual microgravity, to compare the adaptation of non-loaded bones to actual microgravity versus simulated microgravity as reported in literature, and to quantify these changes based on mineral content and bone volume. We hypothesize that microgravity induces adaptive growth in bones that normally do not bear weight, possibly from headward fluid shifts that accompany actual microgravity.

Materials and Methods

Control and Spaceflight Groups

Experimental protocols conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health, and were 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, wild-type C57BL/C mice representing the spaceflight group (n=7) experienced fifteen days of gravity aboard the 15-day NASA shuttle mission STS-131. Eight female wild-type C57-BL/C 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 light-dark 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 NASA-KSC. The calvariae were preserved in liquid nitrogen and shipped 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, wild-type C57BL/C mice. However, only seven were included in the current study. Three of the eleven mice were euthanized one day after landing instead of within four hours. The same number of days also held true for three of the eleven ground control mice, which we

excluded. Additionally, one calvaria from the spaceflight group suffered multiple bone fractures from inappropriate handling and storage, and was deemed unsuitable for analysis.

Micro-Computed Tomography Assessment of Mice Calvariae

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/cm^3) with a beam hardening correction algorithm applied during image reconstruction.

Bone structure and histomorphometric parameters were visualized and determined using Skyscan software, Dataviewer, CTAn (Kontich, Belgium). A volume of interest (VOI, defined by a standardized rectangular volume measuring 18mm^3, 5.5mm × 1.2mm area in the coronal plane, 2.7mm depth) 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 then calculated: total volume (TV, defined as the VOI), bone volume (BV, defined as the volume within the VOI occupied by bone), bone volume fraction (BV/TV, defined as the proportion of the VOI occupied by bone, expressed as a percentage), cross-sectional bone thickness (Cs.Th, measured as the 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 [17]), and tissue mineral density (TMD, determined by calibration of the attenuation values to those obtained from the HA rods).

Statistical Analysis

All 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/TV, Cs.Th and TMD, respectively. Significance was set at $p < 0.05$.

Results

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 cage-loading 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). Previous studies of weight changes in astronauts attribute most of the reduction to fluid loss (60%), with fat utilization (30%) and muscle catabolism (10%) accounting for the remainder [18].

Comparing murine calvariae, BV/TV was increased (p<0.05) from a mean of 9.64 ±0.79% (Mean ± SD) in the ground control group to 10.48 ±0.47% in the spaceflight group (Fig. 4A). Correspondingly, Cs.Th showed a trend of an increase (p=0.12) from a mean of 0.099 ±0.006mm in the ground control group to 0.104 ±0.005mm in the spaceflight group (Fig. 4B). 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. 4C).

Of the three parameters used to determine bone growth, calvaria BV/TV from the spaceflight group exhibited a statistically significant increase of 8.7% (10.48/9.64 – 1) over the ground control group. Cs.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 was also some bone expansion into sinuses within the parietal bones (Fig. 2).

Despite the more aggressive weight-reduction experienced by the spaceflight group (10.76%) compared their ground control littermates (5.77%), the calvariae from the spaceflight group demonstrated volume expansion, which contradicts the observed changes in the bodyweights between the two groups.

Discussion

Our data support the hypothesis that microgravity causes adaptive growth of murine skull bone, reflected by changes in bone volume and possibly bone mineral density. The increase in bone volume reached significance in the mice exposed to microgravity while the change in cross-sectional thickness did not, possibly due to bone expansion into the cavities of the skull and the sagittal suture. However, this trend in cross-sectional thickness may be strengthened by a longer stay in microgravity. Tissue mineral density did not vary between the two groups, but past studies of weight-bearing bones [19] indicate that changes in this parameter are generally seen with much longer duration of spaceflight.

From previous hindlimb unloading studies, we hypothetically attribute the bone remodeling witnessed by us to the head-ward shift of interstitial fluid (ISF) that accompanies microgravity in the absence of gravity in space. Hillsley and Frangos hypothesize that in microgravity, unloading and decreased vascular and ISF flow to weight-bearing bones promotes degeneration while the same fluid forces redistribute to the head to incite growth [20]. Hindlimb-unloading of rats, another ground-based technique to simulate microgravity, causes decreased perfusion of murine distal extremities and a corresponding rostral increase of fluid pressure [21, 22, 23]. By artificially incrementing intramedullary fluid pressure in the femurs of hindlimb-unloaded rats and noting subsequent increases in bone mineral

content and size of the femur, Bergula and co-workers establish the capability of ISF flow to alter bone-remodeling independent of skeletal loading [24]. The same principle may be used to explain the remodeling of calvariae in space.

Normally weight-bearing bones such as in the lumbar spine decrease in mineral density in spaceflight or simulated microgravity, while bones that do not bear weight such as the skull show increased BMD [25]. The majority of studies that have been conducted to determine the physiological consequences of microgravity discuss the changes specifically in bones that normally bear weight. A study analyzing effects of long-duration spaceflight missions (average length of 176 ±45 days) shows substantial reductions in bone mineral density in several weight-bearing bones including the lumbar spine and leg, resulting in a total body BMD decrease of 0.35%/month [19]. The proposed mechanism for this is an uncoupling of normal bone remodeling with increasing bone resorption without compensatory bone formation [26]. It is possible that staying in microgravity for longer duration will results in more significant changes in the skull.

Analyzing calvariae of spaceflight mice allows the comparison with similar studies that use simulated microgravity conditions. Following 17-week horizontal bed rest, several weight-bearing bones including the lumbar spine and the calcaneus suffer mineral losses ranging from 2.2 to 10.4% while an increase of 3.4% is noted in the skull, a finding supported by our data [27]. In the same study, Arnaud and associates implement another flight simulation model with hindlimb unloading by tail suspension to induce a cephalic fluid shift. After 3 weeks of unloading, they determined that bone mineral content is higher in the skull and lower in the hindlimbs. The consistency of our results with these studies is consistent with the ability of bed rest and hindlimb suspension at simulating the effects of microgravity on unloaded bones.

Increases in bone measurements following exposure to microgravity are likely caused by head-ward fluid shifts, resulting in a syndrome similar to idiopathic intracranial hypertension, as previously discussed [28]. Mader and researchers also discuss the role of cephalic fluid shifts in causing jugular vein distension, leading to a reduction in the pressure gradient between cerebrospinal fluid and venous blood flow. The smaller gradient does not allow for sufficient drainage of cerebrospinal fluid, leading to an increase in intracranial pressure.

Our study provides initial insight into the effects of microgravity on non-weight bearing bones. Future studies in this field will include longer flight duration of 30 days on the Russian satellite Bion. However, if limited by lack of spaceflight opportunities, hindlimb unloading of mice and bed rest can serve as valid models for simulating microgravity. By first gathering evidence on the skeletal changes associated with microgravity, it is possible to explore other physiological changes in order to gain a better understanding of the mechanisms that cause skeletal changes during and post spaceflight in astronauts.

Conclusion

Fifteen days of microgravity aboard the STS-131 mission induced adaptive changes in the non-weight-bearing murine calvaria. Of the three parameters measured, there was a statistically significant increase in bone volume, and a trend of increase in cross-sectional thickness. Tissue mineral density remained relatively unchanged.

Acknowledgments

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Highlights

- We examine the effect of microgravity on non-loaded bone

- Fifteen days of spaceflight induces adaptive growth on murine calvaria

- Microgravity promotes significant increase in bone volume

- Microgravity promotes trend of increase in cross-sectional thickness

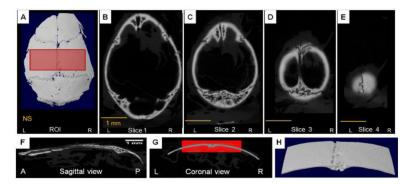- Microgravity does not promote changes in bone mineral density

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 30°.
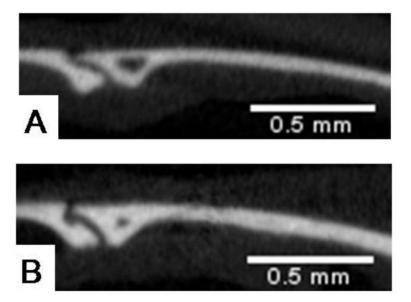
FIGURE 2.
Calvaria from spaceflight group (panel B) showed increase in cross-sectional thickness compared to calvaria from ground control group (panel A). Bone also expanded into the cavity in the parietal bone adjacent to the suture.
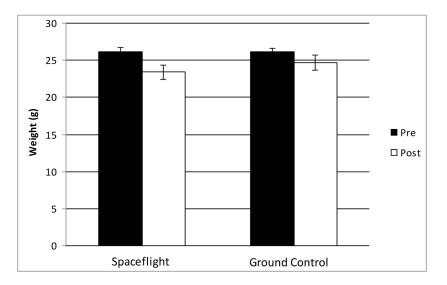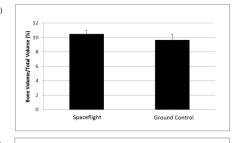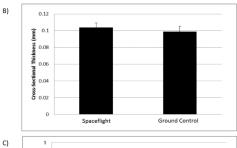
FIGURE 3.
Average weights of the ground control and spaceflight groups before and after fifteen days of cage-loading. Data are mean ± SD; n = 7 in Spaceflight, 8 in Ground Control groups. Fifteen days of microgravity was enough to instigate substantial weight loss in mice. Average weights of the two groups were essentially the same before cage-loading, with the spaceflight group 0.04 grams heavier at 26.20 grams and the ground control group at 26.16 grams. After their respective fifteen days of being caged, the spaceflight group lost an average of 2.82 grams, tantamount to a 10.76% weight reduction. The ground control group only lost an average of 1.51 grams, equal to 5.77% of its pre-caged weight. This striking difference cannot be sufficiently justified by two days of fasting observed in the spaceflight mice at the start of the mission. Continuous systemic response to microgravity including fluid loss, adipose utilization and muscle atrophy from unloading of extremities is a more plausible explanation for the variation between the two groups.
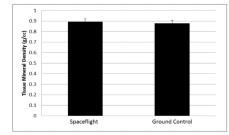
FIGURE 4.
Comparison of ground control and spaceflight groups for bone volume, cross sectional bone thickness, and tissue mineral density. Data are mean ± SD; n = 7 in Spaceflight, 8 in Ground Control groups. Microgravity-induced bone remodeling was reflected by increases in all three parameters we used to define calvaria growth. Compared to their ground control littermates, mice from the shuttle mission showed a relative difference of +8.7% in calvaria bone volume fraction, +5.1% in cross-sectional thickness, and +1.8% in tissue mineral density. Although only the bone volume fraction reached significance, we believe that a mission of longer duration and/or a larger number of samples will better represent the impact of microgravity on unloaded bone (see discussion).