Effects of Spaceflight on Cancellous and Cortical Bone in Proximal Femur in Rats

by
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Abstract approved:_____

Urszula Iwaniec

Abstract

Bone loss is common in astronauts during long-duration spaceflight missions with severe bone loss often occurring in the proximal femur, a region not typically investigated in animal models for microgravity. We recently evaluated bone microarchitecture in the femoral head in rapidly growing male Sprague Dawley rats subjected to a 4-day spaceflight aboard STS-41. Compared to ground controls, cancellous bone volume/tissue volume (BV/TV) was lower in the flight animals. The effects of spaceflight on the rodent skeleton can vary with age, duration of flight, and potentially strain and sex. We therefore evaluated bone in the proximal femur from two additional missions: a 10-day mission (STS-57) with growing male Fisher rats, and a 14-day mission (STS-62) with growing ovariectomized (ovx) Fisher 344 rats. Cancellous microarchitecture and cortical thickness were assessed using microcomputed tomography. In male rats, BV/TV was lower in flight animals compared to flight controls but differences were not significant compared to baseline. In ovx rats, BV/TV was lower in flight animals compared to flight controls and tended to be lower ($p=0.056$)compared to baseline. Cortical thickness did not differ among groups. Taken together, these findings support the conclusion that spaceflight results in cancellous osteopenia in femoral head of growing rats.

Key Words: spaceflight, proximal femur, bone microarchitecture, micro computed tomography, bone volume/tissue volume

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Amanda Gamboa, Author

Introduction

Biological systems evolved in the presence of a nearly uniform gravitational field (Turner et. al., 2000). Not surprisingly, the near absence of 'weight' during orbital spaceflight results in profound adaptive responses in multiple organ systems including the skeleton. Abnormalities in bone and mineral metabolism occurring in astronauts during spaceflight result in net resorption of bone matrix (Turner et. al., 2019). It is unclear whether the changes in bone metabolism during spaceflight are self-limiting. However, evidence to date indicates that long-duration missions will be detrimental to bone health in astronauts.

Bone loss can occur at many skeletal sites but the greatest bone loss detected in astronauts is in the lower body, and in particular the proximal femur (Turner et. al., 2019). Animal studies, primarily using rodents, are important for modeling the effects of spaceflight on the human skeleton. Studies to date have focused on the effects of spaceflight on bone growth and turnover at a variety of weight bearing, load bearing and unloaded skeletal sites (Keune et. al., 2015, Turner et. al., 2000). The majority of spaceflight experiments using rodents were performed prior to widespread availability of micro-computed tomography (μ-CT) (Keune et. al., 2015). Consequently, limited information is available regarding the effects of spaceflight on bone microarchitecture.

The effect of spaceflight on proximal femur has not been explored in detail. We analyzed distal epiphysis, distal metaphysis, diaphysis and proximal region of the femur following a 14-day spaceflight (STS-58) in male rats. Notably, we found that the proximal region of the femur exhibited the largest reduction in mRNA levels for bone matrix proteins (Evans et. al., 1998). Based on this observation, we performed μ-CT analysis of the proximal region of the femoral head of archived bone specimens from rapidly growing male Sprague Dawley rats flown aboard

STS-41 (KSC, L. W.), where we observed strikingly lower cancellous bone volume fraction (BV/TV) in the flight animals. The observed cancellous osteopenia was a surprise because of the short duration (4 days) of the spaceflight mission. To determine whether this finding can be generalized, we performed μ-CT analysis of the femoral head of archived bone specimens from two additional spaceflight experiments, STS-57 and STS-62. STS-57 was a 10-day mission performed using growing male Fisher-344 rats and STS-62 was a 14-day mission using growing ovariectomized (ovx) Fisher 344 rats.

Methods

STS-57 was a Shuttle-SPACEHAB mission of Space Shuttle Endeavour that launched June 21, 1993 from Kennedy Space Center, Florida (KSC, L. W.). 7.5-week-old male Fisher 344 rats were flown on the flight. The experimental protocol was approved by the NASA Animal Care and Use Committee and flight details have been published (Westerlind and Turner 1995). In brief, the rats were randomized into one of three groups, baseline control (baseline, n=6), ground based flight control (flight control, n=12), or spaceflight (flight, n=12). The baseline group was sacrificed on day of launch. The flight animals were flown on the STS-57 flight for 10 days. Flight and flight control animals were housed in animal enclosure modules (AEMs) maintained at 28°C. All animals were provided with food and water *ad libitum*. Flight animals were sacrificed 5-8 hours after landing by decapitation and femora were removed, fixed overnight in 10% formalin, and stored in 70% ethanol for evaluation.

STS-62 was a Space Shuttle program mission flown aboard Space Shuttle Columbia that launched March 4, 1994 (KSC, L. W.). 12-week-old ovariectomized Fisher 344 rats were used in the study. The experimental protocol was approved by the NASA Animal Care and Use Committee and flight details have been published (Cavolina et. Al., 1997). In brief, the rats were

ovx prior to launch and randomized into one of three groups, baseline control (baseline, n=6), ground based-flight control (flight control, n=12), or spaceflight (flight, n=12). The baseline group was sacrificed on day of launch. The flight animals were flown on STS-62 for 14 days. Flight and flight control animals were housed in AEMs maintained at 28°C. All animals were provided with food and water *ad libitum*. Flight animals were sacrificed 4-6 hours after landing. Asynchronous groups of ovary-intact rats were sacrificed to provide age-matched reference values for baseline and flight. The asynchronous component of the study was approved by the Animal Use and Care Committee at the Mayo Clinic (Rochester, MN) where the study was performed. The purpose of the ovary-intact controls was to evaluate normal age related changes in bone over the 14 day flight period and to verify that ovx resulted in cancellous osteopenia. All rats were sacrificed by decapitation and femora were removed, fixed overnight in 10% formalin, and stored in 70% ethanol for evaluation.

Micro-computed Tomography

Bone volume and microarchitecture were assessed using a Scanco μCT scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 12x12x12 microns, 55 kVp X-ray voltage, 145 μA intensity, and 200 ms integration time. Filtering parameters sigma and support were set to 0.8 and 1, respectively. Bone segmentation was conducted at a threshold value of 245 (gray scale, 0-1000), determined empirically. Cancellous bone was evaluated in the femur head. Cortical bone was evaluated in the proximal femur diaphysis.

In both studies, a sample of 20 slices (240 μm) of cancellous bone was assessed in the proximal half of the femoral head as previously described (Turner et. al., 2019). Automated irregular manual contouring was used to delineate cancellous from cortical bone. Direct cancellous bone measurements included cancellous bone volume/tissue volume (%), connectivity

density (mm^{-3}), trabecular thickness (μm), trabecular number (mm^{-1}), and trabecular separation (μm).

In both studies, 63 slices of cortical bone were assessed with the first slice 690 slices distal from the top of femoral head. Evaluation continued 62 slices distally. Direct cortical bone measurements included cortical thickness (μm).

Statistical Analysis

Mean responses were compared between three groups (baseline, ground control, flight) using analysis of variance (ANOVA). Flight rats were compared to baseline and flight control rats using a Dunnett's Posthoc Multiple Comparison test. Data analysis was performed using RStudio version 3.6.1. Data are presented as box and whisker plots. Differences were considered significant at $p \leq 0.05$. Trends are reported when $P < 0.1$.

Results

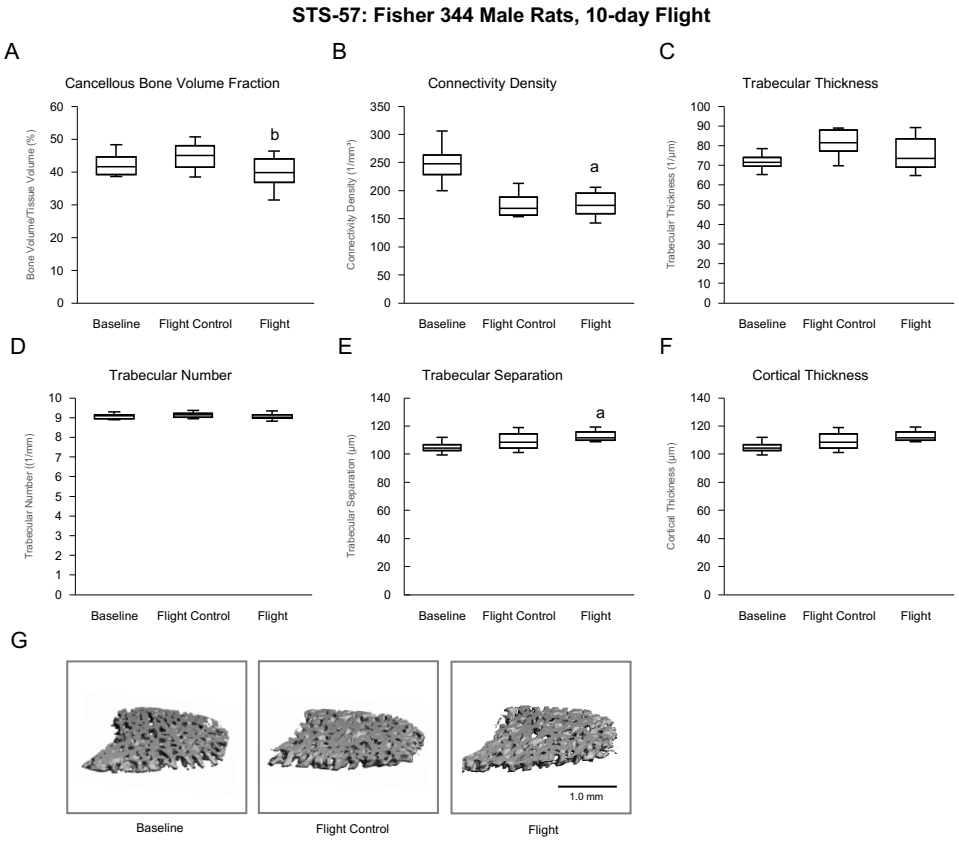

Figure 1

Figure 1. Effects of a 10-day spaceflight aboard STS-57 in growing male Fisher rats on cancellous bone volume fraction (A), connectivity density (B), trabecular thickness (C), trabecular number (D) and trabecular separation (E) in the femoral head, and cortical thickness (F) in the proximal femur diaphysis. Representative three-dimensional images of cancellous bone from animals in each treatment group are shown in panel G. [a]different from baseline, $P < 0.05$, [b]different from flight control, $P < 0.05$.

The effects of spaceflight on cancellous bone microarchitecture in the femoral head in 7.5 week-old Fisher 344 male rats flown on STS-57 for 10 days are shown in **Figure 1 (A-E** and **G)**. Flight rats had lower cancellous bone volume/tissue volume compared to flight controls (**A**). Flight rats had lower connectivity density (**B**) and higher trabecular separation (**E)** compared to baseline rats. Significant differences were not detected between flight and flight control rats for any of the remaining endpoints evaluated. Representative µ–CT images of cancellous bone in the femur from the 3 treatment groups are shown in **Figure 1G**. The effects of spaceflight on cortical

bone microarchitecture in the femoral diaphysis is shown in **Figure 1 (F).** Significant differences in cortical thickness in the proximal femur diaphysis were not detected with treatment.

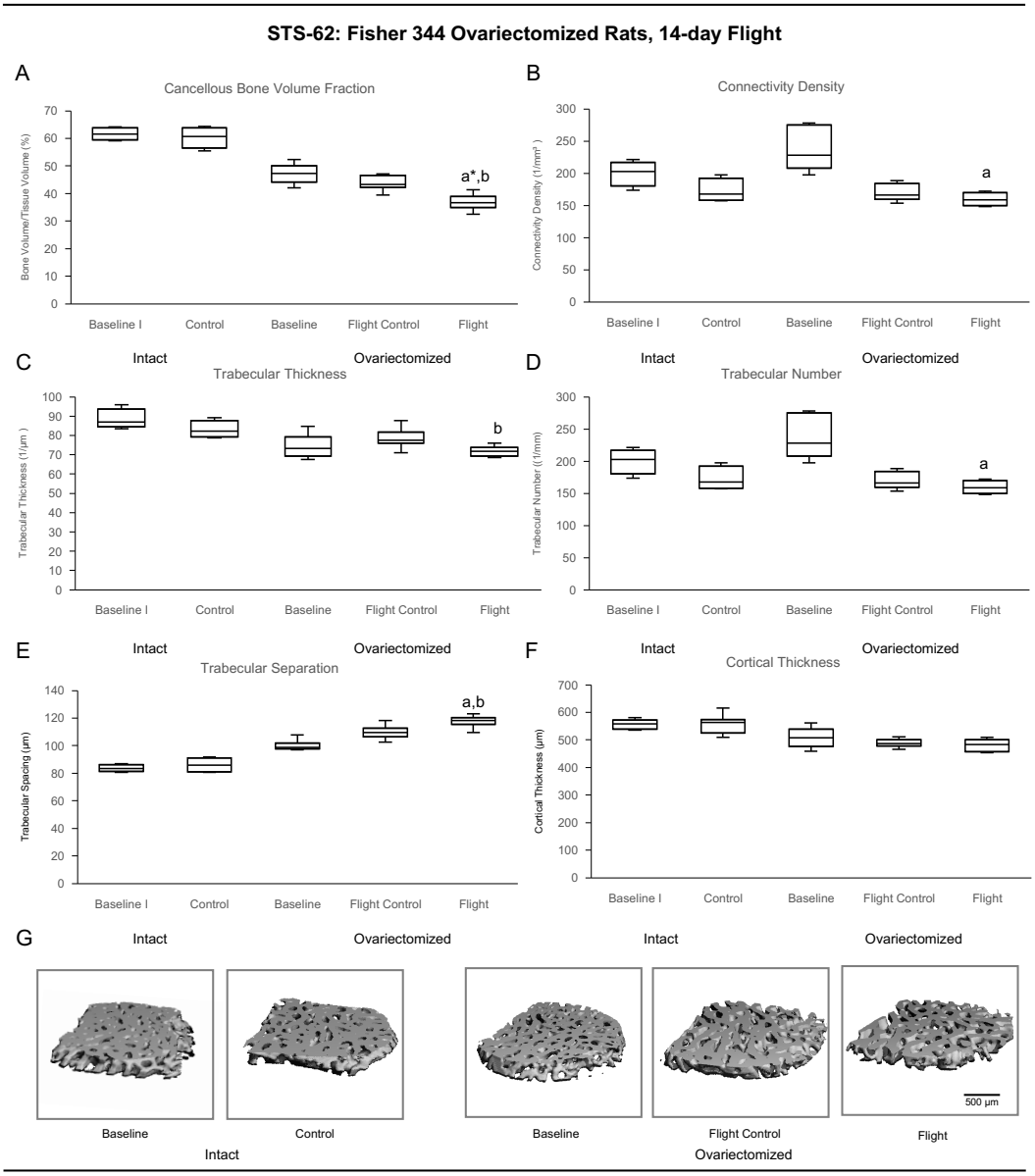

Figure 2

Figure 2. Effects of 14-day spaceflight aboard STS-62 in growing ovariectomized (ovx) Fisher 344 rats on bone volume fraction (A), connectivity density (B), trabecular thickness (C), trabecular number (D) and trabecular separation (E) in the femoral head, and cortical thickness (F) in the proximal femur diaphysis. Representative three-dimensional images of cancellous bone from animals in each treatment group are shown in panel G. Asynchronous groups of age-matched ovary-intact rats (Intact) are shown as reference values for baseline and flight animals. [a]different from baseline, $P < 0.05$, [a*]different from baseline $P < 0.1$, [b]different from flight control $P < 0.05$.

The effects of spaceflight on cancellous bone microarchitecture in the femoral head in 12-week-old ovariectomized rats flown on STS-62 for 14 days are shown in **Figure 2 (A-E** and **G)**. Flight rats tended (P = 0.056) to have lower cancellous bone volume/tissue volume (**A**) and had lower connectivity density (**B**) and trabecular number (**D**) and higher trabecular separation (**E**) compared to baseline rats. Flight rats also had lower bone volume/tissue volume (**A**) and trabecular thickness **(C)**, and higher trabecular separation (**E**) compared to flight control rats. Representative μ–CT images of cancellous bone in the femur from the 3 treatment groups are shown in **Figure 2G**. The effects of spaceflight on cortical bone microarchitecture in the femoral diaphysis are shown **Figure 2F.** Significant differences in cortical thickness in the proximal femur diaphysis were not detected with treatment.

Discussion

Short duration (4 days) spaceflight aboard STS-41 resulted in cancellous osteopenia in the proximal portion of the head of the femur in rapidly growing male Sprague Dawley rats (Turner et. al., 2019). Retrospective analyses of femurs archived from rats flown in low Earth orbit aboard STS-57 and STS-62 presented here provide a more complete picture of the response of the femur head to microgravity. Specifically, cancellous bone volume fraction was lower in flight animals than in age-matched ground controls. However, changes in bone microarchitecture were not uniform among studies.

The proximal femur is clinically relevant but rarely evaluated in animal models. Low trauma fractures are common in the femoral neck of individuals with osteoporosis (Tsuda et. al., 2017). Fractures occur at several locations in the femoral neck, including intertrochanteric, transcervical neck, subcapital neck, and subtrochanteric region. Fractures occurring through the

femoral head are more associated with trauma and are particularly difficult to manage (Scolaro et. al., 2017). That being said, traumatic injury would be the most likely cause of a fracture during a spaceflight mission and a microgravity-induced reduction in bone volume fraction at this site would likely reduce the magnitude of trauma required for fracture.

There is evidence for location-specific (e.g., appendicular versus axial) and compartment-specific (cancellous versus cortical) effects of microgravity on bone in astronauts and rodents (Keune et. al., 2015). Unfortunately, a limitation of the animal work has been variability in the methods used to evaluate the bone response. In general, investigators had limited access to tissues and μCT was not commonly available for evaluation of bone microarchitecture during the interval 1970s-1990s when most spaceflight studies using rats as models were performed (Keune et. al., 2015). In this regard, we have been fortunate in being able to analyze bone architecture in archived femur, humerus, lumbar vertebra and calvaria from rats flown on STS-62 (Keune et al., 2015). Additionally, we have analyzed dynamic and static histomorphometry in tibia and lumbar vertebra in rats from this flight (Cavolina et. al., 1997, Westerlind et. al., 1997, Keune et. al., 2015, Keune et. al., 2016). Compared to ground-based flight controls, flight animals had lower cancellous bone volume fraction in femur at all sites evaluated (head, distal metaphysis and distal epiphysis) but showed no change in cortical thickness (midshaft and proximal femur) (**Table 1**).

Table 1. Effects of spaceflight on cancellous bone volume fraction (femur, humerus, lumbar vertebra) and cortical thickness (femur, humerus) in 12-week-old ovareictomized Fisher rats flown aboard STS-62 for 14 days.

	Flight Control	Flight	% Difference	Reference
Cancellous bone volume fraction (%)				
Femur				
Head	43.9 ± 0.7	36.9 ± 0.9	16.0*	Current study
Metaphysis	6.8 ± 0.3	4.9 ± 0.3	27.9*	Keune et. al., 2016
Epiphysis	28.2 ± 0.2	24.1 ± 0.4	14.5*	Keune et. al., 2016
Humerus				
Epiphysis	32.6 ± 0.5	32.6 ± 0.3	0.0	Keune et. al., 2016
Lumbar vertebra				
Body	20.9 ± 0.4	16.9 ± 0.8	19.1*	Keune et. al., 2016
Cortical thickness				
Femur				
Proximal diaphysis (anterior half)	489 ± 7	482 ± 6	1.4	Current study
Mid-diaphysis	492 ± 4	478 ± 4	2.8	Keune et. al., 2016
Humerus				
Mid-diaphysis	486 ± 4	477 ± 4	1.9	Keune et. al., 2016

*Diffferent from ground-base Flight Control, $P < 0.05$

μ-CT provides limited insight into cellular and molecular mechanisms responsible for the site-specific alterations in bone microarchitecture occurring during spaceflight. Histomorphometric analysis suggests differential responses at cortical and cancellous bone sites. Specifically, longer duration spaceflight (> 7 days) results in decreased periosteal bone formation in weight-bearing bones (Keune et. al., 2015), likely contributing to decreased bone mineral content (Keune et. al., 2015). Reduced bone formation at the periosteum appears to be due, at least in part, to lower mRNA levels for bone matrix proteins (Evans et. al., 1998). In contrast, decreased bone formation has not been established to play an important role in development of cancellous osteopenia during spaceflight. The most detailed rodent spaceflight studies to date were performed on rats flown aboard STS-62. In these animals, cancellous osteopenia resulted from increased bone resorption in tibia and lumbar vertebra, as ascertained by fluorochrome label escape, with no change in bone formation, as ascertained by static and dynamic bone histomorphometry (Turner et. al., 2019). At the molecular level, we observed increased expression levels of two cytokines, interleukin-1beta and interferon gamma, in proximal tibial metaphysis known to increase bone resorption (Zhang et. al., 1998).

In contrast to STS-62, histomorphometric evaluation and gene expression analyses failed to detect spaceflight-induced changes in the skeleton following the 4-day STS-41 flight (Backup et. al., 1994, Turner et. al., 1995, Turner et. al., 1995). Thus, the dramatically lower cancellous bone volume fraction noted in the femoral head of these animals (~46% compared to ground controls) (Turner et. al., 2019), is a surprise. Because the rats were very young and rapidly growing, it is likely that the lower bone volume fraction in the femoral head of flight animals was due to reduced bone accrual. Unfortunately, baseline controls were not available for analysis.

Here, we analyzed femoral heads from rats flown aboard STS-57 and STS-62 to determine whether the results obtained for STS-41 are generalizable. Consistent with the original observation in rats flown aboard STS-41, spaceflight resulted in osteopenia in the femoral head in rats flown both aboard STS-57 and STS-62. However, the magnitude of response was not replicated (**Table 1**). To address the possibility of error, a different observer blinded to treatment re-measured the μ-CT scans of the femoral head in rats flown aboard STS-41. The comparable result of the 2nd analysis excludes measurement error but does not provide additional insight. Based on absence of reductions in longitudinal bone growth, absence in reduction in mRNA levels bone for matrix proteins, and absence of histomorphometric evidence for decreased bone formation at skeletal sites assessed in rats from STS-61, the most likely explanation for the dramatic osteopenia in femoral head is increased bone resorption. It remains unclear, however, whether the more modest osteopenia in rats flown aboard STS-57 and STS-62 reflects differences due to strain, age, duration of flight or other factors. Rats flown aboard STS-57 and STS-62 were 7.5 and 12 weeks old at launch, respectively, compared to 39 days old in STS-41. The marked differences in cancellous architecture in flight animals in these two experiments

compared to baseline suggests that normal changes associated with skeletal maturation (e.g., decreased connectivity density and trabecular number, and increased trabecular separation) may have been accelerated early in spaceflight.

In recent years, the mouse has become the exclusive rodent model flown on spaceflight missions. However, there remains a paucity of publications detailing the skeletal response of mice to microgravity, and to our knowledge, the effect of spaceflight on the femoral head has not been evaluated in mice.

In summary, spaceflight ranging from 4 to 14 days in duration in two strains of growing male rats (Sprague Dawley and Fisher 322) and in growing ovx Fisher 322 rats resulted in osteopenia in the femoral head. It is noteworthy that the most significant bone loss during long duration spaceflight in astronauts is in the proximal femur. Thus, future studies performed in more skeletally mature animals should carefully evaluate this site.

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