Effects of spaceflight on cancellous and cortical bone in proximal femur in growing rats

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ABSTRACT

Mechanical loading of the skeleton during normal weight bearing plays an important role in bone accrual and turnover balance. We recently evaluated bone microarchitecture in the femoral head in 5.6-week-old male Sprague Dawley rats subjected to a 4-day spaceflight aboard STS-41. Compared to weight bearing ground controls, cancellous bone volume fraction was dramatically lower in animals subjected to microgravity. The effects of spaceflight on the rat skeleton are potentially influenced by factors such as age, duration of flight, strain and sex. To test the generalizability of our initial observation, we evaluated archived proximal femora from two additional spaceflight missions: a 10-day mission (STS-57) with 7.5-week-old male Fisher 344 rats, and a 14-day mission (STS-62) with 12-week-old ovariectomized (ovx) female Fisher 344 rats. Cancellous microarchitecture and cortical thickness were assessed using x-ray microtomography/microcomputed tomography. In male rats, cancellous bone volume fraction (bone volume/tissue volume) was lower in flight animals compared to flight controls, differences were not significant compared to baseline. In ovx female rats, cancellous bone volume fraction was lower in flight animals compared to flight controls and baseline, indicating net bone loss. Cortical thickness did not differ among groups in either experiment. In summary, findings from three separate studies support the conclusion that spaceflight results in cancellous osteopenia in femoral head of growing rats.

1. Introduction

Life on Earth evolved in the presence of a nearly uniform gravitational field (Turner, 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 homeostasis in astronauts during spaceflight result in net bone loss (Sibonga, 2013). Although it is unclear whether the changes in bone metabolism are self-limiting, evidence to date in animals and humans indicates that long-duration space missions will be detrimental to bone.

Animal studies, done primarily in rodents, are important for modeling the effects of spaceflight at all stages of skeletal maturation. Studies to date have investigated the effects of spaceflight on bone accrual and turnover balance at a variety of weight bearing, load-bearing, and unloaded skeletal sites in growing rodents (Bikle and Halloran, 1999; Turner, 2000). However, the majority of spaceflight experiments using rodents, primarily rats, were performed prior to widespread availability of x-ray microtomography/microcomputed tomography (μCT) (Keune et al., 2015). Consequently, limited information is available regarding the effects of spaceflight on bone microarchitecture.

The proximal tibia and distal femur are commonly evaluated following spaceflight. In contrast, the effects of spaceflight on proximal femur is much less commonly reported. Bone changes can occur at many skeletal sites but the greatest loss in astronauts is detected in the lower body and, in particular, the proximal femur (Keyak et al., 2009; Lang et al., 2006; Sibonga et al., 2007). In one of the few studies investigating multiple skeletal compartments, we analyzed gene expression in distal epiphysis, distal metaphysis, diaphysis, and proximal region of the femur following a 14-day spaceflight (STS-58) in growing male rats.

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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 initial observation, we evaluated bone architecture (using μCT) in the proximal region of the femoral head of archived bone specimens from rapidly growing male Sprague Dawley rats flown aboard STS-41 (Turner et al., 2019), and observed strikingly lower cancellous bone volume fraction in the flight animals. The cancellous osteopenia was a surprise because of the short duration (4 days) of the spaceflight mission. To determine the generalizability of this finding across sex and strain, we assessed bone microarchitecture in the femoral head in 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 ovarioctomized (ovx) female Fisher 344 rats.

2. Methods

STS-57 was a Shuttle-SPACEHAB mission of Space Shuttle Endeavour. The mission launched June 21, 1993 from Kennedy Space Center, Florida. 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 (Westerdink 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 euthanized on day of launch. The flight animals were flown for 10 days. Flight control rats were housed in Animal Enclosure Modules (AEMs) (Moyer et al., 2016) identical to those housing the flight animals and temperature, humidity and light levels adjusted to values in flight AEMs. All animals were provided with NASA Nutrient-upgraded Rodent Foodbar (NuRFB) (Sun et al., 2010; Tou et al., 2003) and water ad libitum. Flight animals were euthanized 5–8 h 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. 12-week-old ovx female 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 female rats were ovx 14 days 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 euthanized on day of launch. The flight animals were housed in AEMs and flown for 14 days. Flight control rats were housed in AEMs and temperature, humidity and light levels were adjusted to values in flight AEMs. Flight and flight control rats were provided with food (AEM NuRFB) and water ad libitum. Flight animals were euthanized 4–6 h after landing. Asynchronous groups of ovary-intact vivarium-housed rats on a standard rat chow diet were euthanized 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 euthanized by decapitation and femora were removed, fixed overnight in 10% formalin, and stored in 70% ethanol for evaluation.

2.1. 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. 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 fraction (bone volume/tissue volume, %), connectivity density (mm⁻³), trabecular thickness (μm), trabecular number (mm⁻¹), and trabecular separation (μm). In addition, 62 slices (744 μm) of cortical bone were assessed 690 slices (8.3 mm) distal to the top of femoral head and cortical thickness (μm) measured.

2.2. Statistical analysis

Mean comparisons of quantitative variables (e.g., bone volume fraction) for the spaceflight, flight control, and baseline groups were made using separate one-way analysis of variance models for data from male and female rats. The mean structure of both models was specified as α*Spaceflight + β*Flight Control + γ*Baseline. The linear model conditions of normality and equal variance were assessed numerically using Anderson-Darling and Levene’s tests, and graphically using box-plots, dotplots, normal quantile plots, and residual plots. Dunnett’s procedure determined adjusted P-values for comparing mean outcomes of the spaceflight group to both the baseline and flight control groups. All data are reported as mean ± SE with individual values presented. Differences are considered significant at p ≤ 0.05. Data analysis was performed using R version 3.4.3.

3. Results

The effects of spaceflight on cancellous bone microarchitecture in the femoral head and cortical thickness in the proximal femur diaphysis in 7.5-week-old Fisher 344 male rats flown on STS-57 for 10 days are shown in Fig. 1. Flight rats had lower cancellous bone volume fraction compared to flight control rats (A). Flight rats also had lower connectivity density (B) and higher trabecular separation (E) compared to baseline rats. Significant differences among groups were not detected for any of the remaining cancellous endpoints evaluated. Significant differences in cortical thickness were likewise not detected among groups (F). Representative μCT images of cancellous bone in the femur head from the 3 treatment groups are shown in Fig. 1G.

The effects of spaceflight on cancellous bone microarchitecture in the femoral head and cortical thickness in the proximal femur diaphysis in 12-week-old ovx female rats flown on STS-62 for 14 days are shown in Fig. 2. Age-matched (baseline and flight duration) asynchronous ovary-intact controls are shown as reference groups. Flight rats had lower cancellous bone volume fraction (A) and trabecular thickness (C), and higher trabecular separation (E) compared to flight control rats. Flight rats also had lower cancellous bone volume fraction (A) and connectivity density (B) and higher trabecular separation (E) compared to baseline rats. Significant differences in cortical thickness were not detected with treatment (F). Representative μCT images of cancellous bone in the femur head from the treatment groups are shown in Fig. 2G. The comparative effects of the 14-day spaceflight in 12-week-old ovx female rats flown on STS-62 (current and published studies) and in 16-week-old female C57BL6/J mice subjected to 15 days of spaceflight aboard STS-131 (Blaber et al., 2014) on bone microarchitecture at representative sites are shown in Table 1. Cancellous osteopenia was detected in femur (head, metaphysis and epiphysis) and lumbar vertebra (body), but not humerus (epiphysis) in rats and in the femur head in mice. Cortical thickness was unchanged in femur (rats and mice) and humerus (rats).

4. Discussion

Rodents—historically rats and more recently mice—are the primary animal model for investigating the effects and underlying mechanisms of...
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STS-57: Fisher 344 Male Rats, 10-day Flight

Fig. 1. Effects of a 10-day spaceflight aboard STS-57 in growing male Fisher 344 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 on 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. *Different from baseline, \( P < 0.05 \), †Different from flight control, \( P < 0.05 \).

The dramatically lower cancellous bone volume fraction noted in the femoral head of rats flown aboard STS-41 was a surprise (Turner et al., 1995; Turner et al., 1995). Since the rats were very young and rapidly growing, it is likely that the lower cancellous bone volume fraction in the femoral head of the flight animals was due to reduced bone accrual. However, we cannot verify this because baseline controls were not available for analysis.

We analyzed femoral heads from rats flown aboard STS-57 and STS-62 to determine whether the results obtained for STS-41 are generalizable across sex and strain. Consistent with the original observation in male Sprague-Dawley rats flown aboard STS-41, spaceflight resulted in osteopenia in the femoral head in male Fisher 344 rats flown aboard STS-57 and in ovx female Fisher 344 rats flown aboard STS-62. However, the magnitude of response appeared to be smaller in the latter two studies. Compared to ground controls, cancellous bone volume fraction

action of spaceflight on the skeleton. Here we report data showing that spaceflight results in cancellous osteopenia in the proximal femoral head in rapidly growing male Fisher 344 rats and slowly growing ovx female Fisher 344 rats. The observed osteopenia in the femoral head following spaceflight supports the results of a 4-day study performed in very rapidly growing male Sprague-Dawley rats (STS-41). Spaceflight studies performed in C57BL/6 J mice (Blaber et al., 2014) suggest that this response may not be limited to rats.
in flight rats was -46% in STS-41, -11% in STS-57 and -16% in STS-62.
To address the possibility of error in the initial analysis, a different
observer blinded to treatment re-measured the μCT scans of the femoral
head in rats flown aboard STS-41. The comparable results of the 2nd
analysis excludes measurement error but does not provide additional
insight. Based on absence of reduction in longitudinal bone growth,
absence of reduction in mRNA levels for bone matrix proteins, and
absence of histomorphometric evidence for decreased bone formation at
multiple skeletal sites assessed, the most likely explanation for the
dramatic osteopenia in femoral head in rats from STS-41 is increased
bone resorption (Backup et al., 1994; Turner, 1995; Turner et al., 1995).
It remains unclear, however, whether the more modest osteopenia in
rats flown aboard STS-57 and STS-62 compared to their respective flight
controls reflects differences due to strain, age, duration of flight, or other
factors. However, it is notable that BV/TV in ovx female rats flown
aboard STS-62 was lower than baseline controls, indicating net bone loss
contributed to the osteopenia.

There is evidence for location-specific (e.g., appendicular versus
axial) and compartment-specific (e.g., cancellous versus cortical) effects
of microgravity on bone microarchitecture (Keune et al., 2015). Mech-
anistic inference is challenging because of the limited number of studies
and variation in experimental design and endpoints used to evaluate the
bone response to spaceflight (Turner, 2000). Variables in design include
age at launch, strain, duration of flight, housing conditions during flight,
and interval of time between reloading and necropsy (Morey-Holton
et al., 2000; Vico et al., 1993; Wronski et al., 1987; Zerath et al., 2000).
In general, investigators have had limited access to tissues (often a single
bone). As for endpoints, histomorphometric analysis was the principle
method available for evaluation of bone architecture during the interval
when most spaceflight studies using rats as models were performed
(1970s–1990s) (Keune et al., 2015). In this regard, we have been very
fortunate in being able to analyze archived femur, humerus, lumbar
vertebra and calvarium from ovx female Fisher 344 rats flown on STS-62
(Keune et al., 2015). Additionally, we measured dynamic and static

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**Fig. 2.** Effects of a 14-day spaceflight aboard STS-62 in growing ovariectomized (ovx) female 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 on 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 ovari-intact rats (Intact) are shown as reference values for baseline and flight animals. *Different from baseline, P < 0.05, †Different from flight control, P < 0.05.
and distal epiphysis) but showed no change in cortical thickness. These ground-based flight controls, flight animals had lower cancellous bone volume fraction (femur) and cortical thickness (femur) in 16-week-old female C57BL/6J mice flown aboard STS-131 for 15 days.

Specifically, spaceflight resulted in decreased periosteal bone formation (Cavolina et al., 1997; Keune et al., 2015; Keune et al., 2016; Westerlind et al., 2016). Histomorphometry in tibia and lumbar vertebra in rats from this flight have recommended changes in BV/TV in femoral head (Blaber et al., 2014) that were similar in magnitude to changes observed in ovx female Fisher 344 rats following a 14 day spaceflight. In the ovx female rats, cancellous osteopenia in the flight animals was primarily due to lower trabecular thickness, whereas in ovari-intact mice both trabecular number and thickness were lower in flight animals compared to ground controls.

Table 1

| Flight control | Flight | % difference | Reference |
|----------------|--------|--------------|-----------|
| Ovariectomized female Fisher 344 rats | 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 Humerus | 28.2 ± 0.2 | 24.1 ± 0.4 | 14.5* | Keune et al., 2016 |
| Epiphysis Vertebra | 32.6 ± 0.5 | 32.6 ± 0.3 | 0.0 | Keune et al., 2016 |
| Body | 20.9 ± 0.4 | 16.9 ± 0.8 | 19.1* | Keune et al., 2016 |
| Cortical thickness (μm) | | | |
| Femur Proximal diaphysis | 489 ± 7 | 482 ± 6 | 1.4 | Current study |
| Mid-diaphysis Humerus | 492 ± 4 | 478 ± 4 | 2.8 | Keune et al., 2016 |
| Mid-diaphysis Humerus | 486 ± 4 | 477 ± 4 | 1.9 | Keune et al., 2016 |
| Female C57BL/6J mice | Cancellous bone volume fraction (%) | | |
| Femur Head | 47.0 ± 0.7 | 38.8 ± 1.3 | 17.5* | Blaber et al., 2014 |
| Cortical thickness (μm) | | | |
| Femur Neck | 140 ± 1 | 138 ± 1 | 1.0 | Blaber et al., 2014 |

* Different from flight control, P < 0.05.

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 (Blaber et al., 2014; Gerhaix et al., 2017; Smith et al., 2020; Tavella et al., 2012). Smith et al. reported osteopenia in the distal femur of growing female BALB/c mice following spaceflight (Smith et al., 2020). As mentioned, Blaber et al. reported changes in BV/TV in femoral head of 16-week-old female C57BL/6J mice following a 15-day spaceflight (Blaber et al., 2014) that were similar in magnitude to changes observed in ovari female Fisher 344 rats following a 14 day spaceflight. In the ovari female rats, cancellous osteopenia in the flight animals was primarily due to lower trabecular thickness, whereas in ovari-intact mice both trabecular number and thickness were lower in flight animals compared to ground controls.

The proximal femur is clinically relevant but not commonly evaluated in animal models. Low trauma fractures are common in the femoral neck of individuals with osteoporosis (Tsuda, 2017). Fractures occur at several locations in the proximal femur, including intertrochanteric region, transcervical neck, subcapital neck, and subtrochanteric region. Fractures occurring through the femoral head, the site investigated in this study, are more typically associated with trauma and are particularly difficult to manage (Scolaro et al., 2017). That being said, traumatic injury would be the likely cause of 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. This conclusion is supported by studies demonstrating that spaceflight and simulated spaceflight result in lower mechanical competence (Patterson-Buckendahl et al., 1987; Shirazi-Fard et al., 2015; Spengler et al., 1983; Zernicke et al., 1990).

In summary, spaceflight ranging from 4 to 14 days in duration in two strains of growing male rats (Sprague Dawley and Fisher 344) and in growing ovari female Fisher 344 rats resulted in osteopenia in the femoral head. It is noteworthy that significant bone loss during long duration spaceflight in astronauts occurs in the proximal femur. Thus, future studies performed in skeletally-mature animals should carefully evaluate this clinically relevant site.

Declaration of interest

The authors have no conflict of interest.

CRediT authorship contribution statement

Conceptualization: RT
Data Collection: AG, DO, LS
Data analysis: AB
Drafting manuscript: AG
Revising manuscript content: RT, AB, DO, LS, UI, and AG
Approving final version: RT, AB, DO, LS, UI, and AG
RT takes responsibility for the integrity of the data.

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