Increased bone marrow adiposity in murine femoro-tibial epiphyses exposed to 30 days of microgravity

Jamie Endicott, Jamie Fitzgerald

Bone and Joint Center, Henry Ford Hospital System; Bone and Joint Center, Henry Ford Health System

Abstract

Bone marrow mesenchymal stem cell (BM-MSC) differentiation in long bones is sensitive to mechanical loading. Increased loading promotes osteogenesis and reduces adipogenesis while reduced loading tips MSC differentiation away from the bone formation in favor of adipogenesis. To examine the effects of the unloading on epiphyseal bone and adipocyte content, subchondral femoro-tibial bones isolated from mice flown for 30 days in microgravity were assessed for evidence of altered bone area and adipocyte number. Consistent with the known response of bone to microgravity, 30 days of spaceflight resulted in approximately 25% less subchondral bone area. Concurrently, 10-fold more adipocytes were present in the bone marrow cavities of femur and tibia in flight compared to ground control samples. These data support the hypothesis that biomechanical unloading promotes adipogenic differentiation and confirms earlier studies in rat vertebrae of increased adipogenesis during 14 days of microgravity. The potential long-term effects of increased bone marrow adipocyte formation on flight personnel health is unknown and warrants further investigation.

Introduction

Humans have experienced extended periods of time in the biomechanically unloaded environment of microgravity since the mid–1970s. It has become clear from human, animal and *in vitro* studies that the lack of gravity has detrimental effects on many tissues at the cellular level. These well-documented effects on the musculoskeletal system include bone demineralization and skeletal muscle atrophy [1] [2]. This tissue loss is transient; upon return to normal gravity the lost bone and muscle recovers with eventual restoration of pre-flight tissue architecture and function [3] [4] although some studies argue for incomplete recovery [5] [6] [7]. The degradative changes in muscle and bone changes are due to the aberrant function of existing cells and potentially, altered signaling activity of bone marrow mesenchymal cells (BM-MSCs), which give rise to skeletal tissues. While the effects of microgravity on mature musculoskeletal cell types are well-studied, the effects on BM-resident precursor cells is poorly understood.
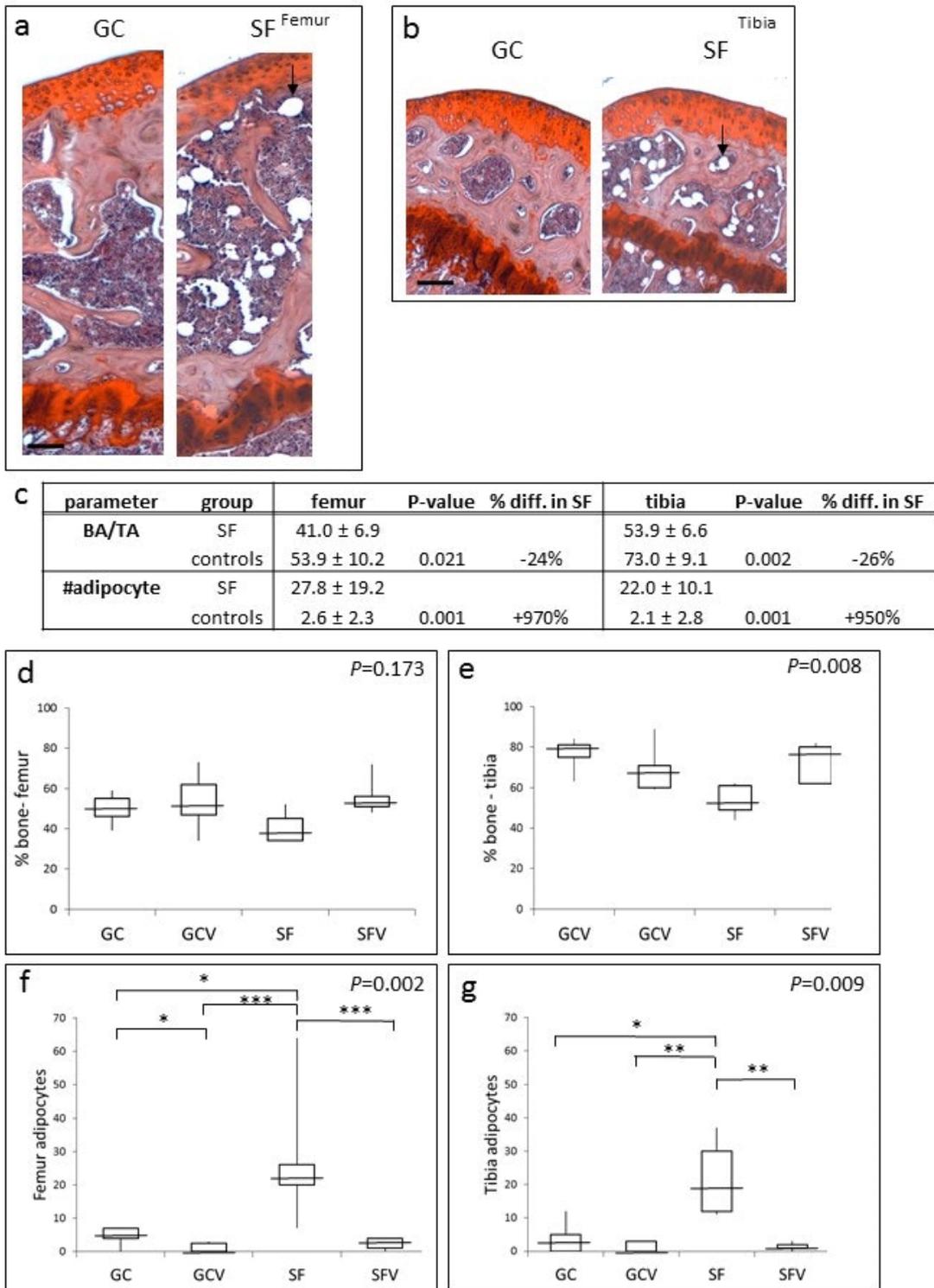
Several studies have reported changes in MSC behavior under conditions of simulated microgravity in rotating wall vessel bioreactors, 2D-clinostats and other devices that neutralize the gravity vector (review article by Ulbrich et al. [8]). While these have provided valuable details on the response of cell and tissues to simulated microgravity, the cells still function within Earth's gravitational field and an accurate understanding of the effects of lack of gravity on BM-MSCs can only be gained in orbit. Several *in vitro* and animal studies into BM-MSC function have been conducted in spaceflight. BM-MSCs flown for 9 days in a 3D-culture system showed decreased cell proliferation and dysregulation of MSC gene expression involved in cell-cycle control [9]. Recent *in vitro* studies on a recoverable orbiting satellite showed that human MSCs cultured for up to 7 days under osteogenic conditions resulted in reduced osteogenesis and enhances adipogenesis [10]. Microgravity suppressed the transcription of genes required for osteogenesis (*RUNX2* and *BMP-2*), increased those for adipogenesis (*p38 MAPK*) and decreased the expression of an adipogenic repressor (*TRIB3*). Only 3 studies have examined bone marrow adiposity *in vivo* in spaceflight. 18.5 days of spaceflight resulted in a decrease in mineralized tissue and an increase in the fat content of the proximal tibia and humerus of Wistar rats [11]. Additional observations suggest that the loss of

bone is due to inhibited bone formation together with a constant rate of bone resorption. Bone loss and increased adiposity were reported in vertebral bodies of ovariectomized rats exposed to 14 days of microgravity [12]. Here, bone cancellous bone area decreased by 1/3 and adipocyte number increased 2-fold. The decrease in overall bone was attributed to increased bone resorption rather than decreased bone formation. Studies on the long bones of mice from the same BION-M1 mission described in the present study showed decreased metaphyseal trabecular bone volume, increased bone resorption and increased apiposity [7]. Interestingly, associated with these changes was decreased osteocyte lacunae volume with multiple empty lacunae present suggesting that spaceflight-induced bone loss is related to osteocyte death [7].

As part of a long-term effort to understand the effects of spaceflight on the musculoskeletal system, we sought to define the effects of microgravity on the differentiation of cells within the BM space. We show increased adiposity with some evidence of decreased bone area within the epiphyseal region. These data confirm and extend findings from earlier studies and indicate that MSC pro-adipogenic pathways continue to be stimulated four weeks into spaceflight.

Objective

To investigate the effects of 30 days of spaceflight on BM-MSC adipogenesis in the long bones of skeletally-mature mice.

parameter	group	femur	P-value	% diff. in SF	tibia	P-value	% diff. in SF
BA/TA	SF	41.0 ± 6.9			53.9 ± 6.6		
	controls	53.9 ± 10.2	0.021	-24%	73.0 ± 9.1	0.002	-26%
#adipocyte	SF	27.8 ± 19.2			22.0 ± 10.1		
	controls	2.6 ± 2.3	0.001	+970%	2.1 ± 2.8	0.001	+950%

Figure Legend

Figure 1.

Sub-chondral bone analysis. Images showing the region of secondary ossification in

femoral condyle **(A)** and tibia **(B)** surfaces of flight and ground control knee samples stained with Safranin-O and counterstained with hematoxylin. Scale bars are 100 mM. Comparison of bone (bone area/total area) and adipocyte numbers in SF and combined ground control samples **(C)**. *P*-values are the Mann-Whitney U test (2-tailed). Errors are s.d. Bone percentage measurements were made for the femur **(D)** and tibia **(E)** for SF and non-flight controls (GC, GCV, and SFV) separately. Adipocyte numbers were calculated for the same joints and experimental groups **(F & G)**. Median, maximum score, minimum score, and 25th and 75th percentile of the bone area and adipocyte number are plotted for each experimental group. Omnibus Kruskal-Wallis *P*-values are shown in the top right-hand corner of each graph. Post-hoc adjustments were conducted on adipocyte number data and *P*-values for pairwise comparisons of experimental groups are indicated. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results & Discussion

We explored the effect of extended microgravity on bone marrow mesenchymal stem cell (BM-MSC) differentiation *in vivo* in femoro-tibial joints isolated from mice exposed to 30 days of microgravity. Representative images from femoral and tibial bones isolated from spaceflight (SF) and ground control (GC) joints are shown in figures. 1A and B. Quantitation of the bone area in sectioned tissue revealed that SF samples had approximately 25% less mineralized bone area in femoral and tibial subchondral regions compared to the combined ground controls (average of GC, GCV, and SFV) (Fig. 1C). When the SF samples are compared with each control separately, box-whisker plots show that tibial metaphyseal bone area is reduced in SF compared to controls (Fig. 1E). This finding of metaphyseal bone loss is consistent with the well-established bone loss associated with microgravity where the bone volume fraction was estimated to be approximately 0.5–1% per day of microgravity exposure in mice [13]. The corresponding measure for humans is the loss of 1–2% BMD per month [14]. The overall loss of bone suggests less bone formation, more bone resorption, or a combination of both. A study on the effect of 14 days of microgravity in rat vertebra indicated the lost bone was a result of increased resorption rather than decreased bone formation. In contrast, in mice flown for 30 days increased bone resorption in trabecular femur and vertebrae were reported [7]. The mice in our study were not assessed for osteoclast activity and we cannot confirm whether bone resorption is compromised. This is important to address because the use of bisphosphonates, which primarily target bone-resorbing osteoclasts, is being explored as a countermeasure for spaceflight-related bone loss [15].

We observed that SF samples also had multiple large vacuoles in both femoral condyle and tibial plateau regions compared to virtually none in the non-flight samples (Fig. 1A & B, arrows). Based on the size, appearance, and location we conclude that these spaces represent adipocytes whose fat fraction has been lost due to the processing for paraffin sections. Strikingly, the SF samples had 10-fold more adipocytes than the combined control samples in both femoral and tibial regions (Fig. 1C). When each group was compared pairwise, the SF samples had significantly more adipocytes than all control groups in both femoral head and tibial plateau (Fig. 1F & G). Increased adipogenesis has been observed in rat vertebrae [12] and long bones [11] exposed to 14 and 18 days of microgravity, respectively, and in the metaphysis of mice exposed to 30 days of microgravity [7]. These findings together with ours are consistent with *in vitro* studies on cultured MSCs from patients with osteoporosis, and rat *in vivo* studies of prolonged mechanical unloading [16] [17] where increased BM adipogenesis has been reported. Collectively, these studies support the hypothesis that BM-MSC adipogenic differentiation is acutely sensitive to bone loading such that under conditions of unloading, adipogenesis is stimulated. The issue of whether osteogenesis is correspondingly compromised *in vivo* is an open question. The molecular details responsible are poorly understood but human BM-MSCs cultured during 12 days of microgravity were shown to downregulate key drivers of osteogenesis such as *Runx2* and *BMP-2* and increased adipogenic markers such as *p38 MAPK* [18]. Additional gene expression, proteomic and epigenetic studies of *in vivo* flight samples are required for a more complete understanding of the major players involved in the altered response of BM-MSCs to microgravity.

Conclusions

The major conclusion of this study is that microgravity has a dramatic effect on MSC pathways to favor adipogenesis in the epiphysis of murine long bones. This observation is consistent with the *in vivo* findings of Keune et al. [12] and Jee et al. [11] of increased adiposity in rodents exposed to short periods of microgravity, and is in agreement with the study of Gerbaix et al. [7] showing increased adipocyte numbers in the femoral metaphysis in mice exposed to the same duration of microgravity. If the same process occurs in humans then it is unknown whether the increased BM adiposity persists upon return to normal gravity and whether the long-term health of flight personnel is compromised. We argue, therefore, that maintenance of biomechanical loading during spaceflight to minimize changes in BM-MSC differentiation pathways is desirable.

Limitations

With the 4 experimental groups having N's between 5 and 7, one limitation is the small experimental numbers when comparing between groups. However, the small numbers were mitigated by the large effect size for adipocyte number which revealed statistical significance when non-parametric testing was used. In addition, since the bones were not labeled with calcein, nor TRAP-stained for osteoclasts, it is not possible to determine whether differences in the bone formation or resorption were responsible for the reduced bone surface area in spaceflight. This will be important to understand if effective countermeasures for bone loss are to be developed.

Alternative Explanations

Conjectures

Several interesting questions arise that are testable on orbiting platforms given the existence of good morphological, biochemical and molecular read-outs for MSC differentiation.

1) Does a biochemical or gravitational threshold exist before altered MSC differentiation is triggered? The availability of rodent facilities and on-board centrifuges designed to spin at fractional gravity on the International Space Station could address this question.

2) What are the molecular pathways involved in altered BM-MSC differentiation due to bone unloading? Insights into molecular mechanisms of BM-MSC differentiation may lead to new therapies for disorders of bone fragility on the Earth.

3) What are the long-term effects of increased BM adipogenesis to human health? While the microgravity-induced bone loss is a major concern for human health, there may be additional health consequences due to the increased BM adiposity. These could potentially include effects of adipocyte-driven crowding of other bone marrow residents such as hematopoietic precursor cells.

Additional Information

Methods

Flight details

Male C57BL/6N mice were flown on the unmanned BION-M1 biosatellite at an altitude of 560–580 km for 30 days between April 19, and May 19, 2013 [18]. Joint tissues were acquired from 6 flight mice as part of NASA's Biospecimen Sharing Program. The mice were specific pathogen-free and 19–20 weeks old at the time of launch and start of asynchronous control experiments. Joint issues were dissected from flight mice in

Moscow 13–16.5 h after landing in Kazakhstan. In addition to the spaceflight mice (SF), there were 8 'flight' vivarium male ground control mice (SFV), 7 asynchronous ground control males (GC), and 7 asynchronous vivarium ground control males (GCV) as determined by agreements between the Russian Space Agency and NASA. The asynchronous control mice were housed 2 months after the flight mission in the same flight habitats for 30 days under environmental conditions simulating the flight conditions including temperature, humidity and gas composition. Since the (asynchronous) control mice arm of the experiment was conducted 3 months after the flight arm of the experiment, the vivarium control cohorts were included concurrently with flight and asynchronous control experiments as reported by Andreev-Andrievskiy et al [18].

Dissection and tissue preparation

Each hind-limb was cut mid-femur and mid-tibia and the left limbs placed in 10% neutral-buffered formalin (NBF). The tissue surrounding the femoro-tibial and sternocostal joints were removed immediately and, following an additional 2 days fixation with 10% NBF, the joints were decalcified in 5% formalin containing 10% formic acid for 2 days at 4℃. The knees were bisected down the trochlear groove following decalcification using a razor blade. The medial halves were paraffin embedded, mounted in paraffin blocks and sections cut (10 mm) using standard methods. The remaining halves were placed in 1× phosphate-buffered saline (PBS) containing 0.01 M ethylenediaminetetraacetic acid (EDTA) at room temperature for 4–6 h and then stored in 70% ethanol. Joint samples were sectioned in the sagittal plane.

Histology

Following dewaxing, every second slide was stained concurrently for Safranin-O (Saf-O) and counterstained with hematoxylin in parallel to visualize the cartilage-subchondral bone boundary and bone marrow space using a protocol from the University of Rochester, Center for Musculoskeletal Research, (https://www.urmc.rochester.edu/musculoskeletal-research/core-services/histology/protocols.aspx). Images were acquired on a Nikon Eclipse TI microscope using a Media Cybernetics Evolution MP camera and ImagePro Plus software. All images are 2560×1920 pixels so at 10X magnification, the scale is approximately 0.11 mm² per pixel.

The extent of subchondral bone was analyzed using ImageJ software and the relative proportions of bone and bone marrow space determined within the secondary ossification center of the femoral condyle and proximal tibia. The proportion of bone was calculated relative to the total area of the secondary ossification center and the number of adipocytes per field within the ossification center calculated.

Statistical analyses

For the femoro-tibial joint histological scoring analysis, measurements were taken from 6 GC, 7 GCV, 5 SF, and 6 SFV samples where good joint histology in the correct plane was obtained. Sections were from the same relative position through the joint centered in the medial femoral condyle. The Mann-Whitney U test was used to compare SF (n=5) bone fraction and adipocyte number with the combined control groups (GC, GCV, SFV) (n=19)(see Fig. 1C). First, omnibus Kruskal-Wallis P-values for multiple independent samples were calculated to determine whether differences exist between experimental groups (top right in each graph). Then, the Kruskal-Wallis test was used pairwise to determine whether differences exist between SF and each of the separate experimental groups (GC, GCV, SFV) [19]. Post-hoc testing by Conover of all pairwise combinations of experimental groups was used to determine which pairs were different for adipocyte number [20]. P-values were adjusted according to the family-wise error rate of Holm and then by the Benjamini-Hochberg FDR method [21]. Median, maximum score, minimum score, and 25th and 75th percentile for the bone area and adipocyte data plotted for each experimental group are shown in figure 1D–G.

Ethics Statement

Animal ethics/IACUC approval for this study was obtained from the MSU Institute of Mitoengineering and of the Biomedical Ethics Commission of IMBP and the study was conducted in compliance with the European Convention for the Protection of Vertebrate animals used for Experimental and Other Scientific purposes.

Citations

[1] Geert Carmeliet, Laurence Vico, and Roger Bouillon. "Space flight: A challenge for normal bone homeostasis". In: *Critical Reviews in Eukaryotic Gene Expression* 11.1-3 (2001), pp. 131–144. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve%5C&db=PubMed%5C&dopt=Citation%5C&list_uids=11693958.

[2] E. A. Ilyin and V. S.Oganov. "Microgravity and musculoskeletal system of mammals". In: *Advances in Space Research* 9.11 (1989), pp. 11–19.

[3] Michael W.C. Payne, David R. Williams, and Guy Trudel. "Space Flight Rehabilitation". In: *American Journal of Physical Medicine & Rehabilitation* 86.7 (2007), pp. 583–591. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve%5C&db=PubMed%5C&dopt=Citation%5C&list_uids=17167347.

[4] J. M. Vogel and M. W. Whittle. "Bone mineral changes - The second manned Skylab mission". In: *Aviation, Space, and Environmental Medicine* 47.4 (1976), pp. 396–400. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve%5C&db=PubMed%5C&dopt=Citation%5C&list_uids=1275827.

[5] J.D. Sibonga et al. "Recovery of spaceflight-induced bone loss: Bone mineral density after long-duration missions as fitted with an exponential function". In: *Bone* 41.6 (2007), pp. 973–978. URL: http://www.ncbi.nlm.nih.gov/pubmed/17931994.

[6] Per A. Tesch et al. "Effects of 17-day spaceflight on knee extensor muscle function and size". In: *European Journal of Applied Physiology* 93.4 (2005), pp. 463–468. URL: http://www.ncbi.nlm.nih.gov/pubmed/15517339.

[7] Maude Gerbaix et al. "One-month spaceflight compromises the bone microstructure, tissue-level mechanical properties, osteocyte survival and lacunae volume in mature mice skeletons". In: *Scientific Reports* 7.1 (2017), p. 2659. DOI: 10.1038/s41598-017-03014-2. URL: https://doi.org/10.1038/s41598-017-03014-2.

[8] Claudia Ulbrich et al. "The Impact of Simulated and Real Microgravity on Bone Cells and Mesenchymal Stem Cells". In: *BioMed Research International* 2014 (2014), p. 928507. URL: https://www.ncbi.nlm.nih.gov/pubmed/25110709.

[9] Massimiliano Monticone et al. "Activation of nervous system development genes in bone marrow derived mesenchymal stem cells following spaceflight exposure". In: *Journal of Cellular Biochemistry* 111.2 (2010), pp. 442–452. URL: https://www.ncbi.nlm.nih.gov/pubmed/20658479.

[10] Cui Zhang et al. "Space microgravity drives transdifferentiation of human bone marrow–derived mesenchymal stem cells from osteogenesis to adipogenesis". In: *The FASEB Journal* 32.8 (2018), pp. 4444–4458. URL: https://www.ncbi.nlm.nih.gov/pubmed/29533735.

[11] W. S. Jee et al. "Effects of spaceflight on trabecular bone in rats". In: *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 244.3 (1983), R310–R314. URL: https://www.ncbi.nlm.nih.gov/pubmed/6829790.

[12] Jessica A Keune et al. "Spaceflight-induced vertebral bone loss in ovariectomized rats is associated with increased bone marrow adiposity and no change in bone formation". In: *NPJ Microgravity* 2 (2016), p. 16016. URL: https://www.ncbi.nlm.nih.gov/pubmed/28725730.

[13] Elizabeth A. Blaber et al. "Microgravity Induces Pelvic Bone Loss through Osteoclastic Activity, Osteocytic Osteolysis, and Osteoblastic Cell Cycle Inhibition by CDKN1a/p21". In: *PLOS One* 8.4 (2013), e61372. URL: https://www.ncbi.nlm.nih.gov/pubmed/23637819.

[14] A. LeBlanc et al. "Bone mineral and lean tissue loss after long duration space flight". In: *Journal of Musculoskeletal & Neuronal Interactions* 1.2 (2000), pp. 157–160. URL: https://www.ncbi.nlm.nih.gov/pubmed/15758512.

[15] A. LeBlanc et al. "Bisphosphonates as a supplement to exercise to protect bone during long-duration spaceflight". In: *Osteoporosis International* 24.7 (2013), pp. 2105–2114. URL: https://www.ncbi.nlm.nih.gov/pubmed/23334732.

[16] Majd Zayzafoon, William E. Gathings, and Jay M. McDonald. "Modeled Microgravity Inhibits Osteogenic Differentiation of Human Mesenchymal Stem Cells and Increases Adipogenesis". In: *Endocrinology* 145.5 (2004), pp. 2421–2432. URL: https://www.ncbi.nlm.nih.gov/pubmed/14749352.

[17] Souhila Ahdjoudj et al. "Transforming Growth Factor β2 Inhibits Adipocyte Differentiation Induced by Skeletal Unloading in Rat Bone Marrow Stroma". In: *Journal of Bone and Mineral Research* 17.4 (2002), pp. 668–677. URL: https://www.ncbi.nlm.nih.gov/pubmed/11918224.

[18] Alexander Andreev-Andrievskiy et al. "Mice in Bion-M 1 Space Mission: Training and Selection". In: *PLOS One* 9.8 (2014), e104830. URL: http://www.ncbi.nlm.nih.gov/pubmed/25133741.

[19] William H. Kruskal and W. Allen Wallis. "Use of Ranks in One-Criterion Variance Analysis". In: *Journal of the American Statistical Association* 47.260 (1952), pp. 583–621.

[20] William J Conover. "Practical nonparametric statistics". In: *Wiley, New York* (1971), p. 474.

[21] Yoav Benjamini and Yosef Hochberg. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing". In: *Journal of the Royal Statistical Society: Series B (Methodological)* 57.1 (1995), pp. 289–300. URL: https://www.jstor.org/stable/2346101.