Food restriction and simulated microgravity: effects on bone and serum leptin

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1Department of Health and Kinesiology and 2Intercollegiate Faculty of Nutrition, Texas A&M University, College Station, Texas; and 3Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana

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Baek K, Barlow AA, Allen MR, Bloomfield SA. Food restriction and simulated microgravity: effects on bone and serum leptin. J Appl Physiol 104: 1086–1093, 2008. First published February 14, 2008; doi:10.1152/japplphysiol.01209.2007.—Leptin is responsible for linking energy metabolism to bone mass. Because astronauts are commonly in negative energy balance during spaceflight, this study was designed to assess individual and combined effects of food restriction and simulated microgravity on bone mass and serum leptin. Six-month-old male Sprague-Dawley rats were randomly assigned to four groups (n = 12 each): two hindlimb-unloading (HU) groups fed 100% (HU100) and 70% (HU70) and two cage-activity control (CC) groups fed 100% (CC100) and 70% (CC70) of their baseline food requirement. After 28 days, CC100 rats gained body weight, whereas all other groups lost body weight; this loss was greater in HU70 than in CC70 and HU100 rats. Serum leptin decreased in CC70 and HU100 (−60% and −27%, respectively) and was not detectable in HU70 animals. Percent osteoid surface in CC70 and HU100 was lower than that of CC100 (7.80%, 8.60% vs. 10.70%, respectively), and this decrease was more pronounced in HU70 animals (4.38%). Mineral apposition rate of CC70, HU100, and HU70 rats was lower than that of CC100 (1.5, 1.6, and 1.5 vs. 2.1 µm/day, respectively). Bone formation rate of CC70, HU100, and HU70 rats was lower than that of CC100 (13.4, 13.1, and 12.2 vs. 40.8 mm²·mm⁻²·day⁻¹, respectively). The change in bone formation rate was correlated with the change in serum leptin value over 28 days (r² = 0.69, P = 0.0007). We conclude that moderate caloric restriction may cause bone loss at susceptible bone sites to a similar degree as does the unloading effect of microgravity; serum leptin may be an important endocrine regulator contributing to this change in skeletal integrity.

THE EFFECTS OF MICROGRAVITY OR DISUSE ON WEIGHT-BEARING BONES HAVE BEEN WELL DOCUMENTED. ABOUT 1% OF BONE MINERAL DENSITY (BMD) IS LOST PER MONTH WHILE IN MICROGRAVITY, ALTHOUGH THE MAGNITUDE OF LOSS IS HIGHLY VARIABLE AMONG SUBJECTS AND AMONG ANATOMIC SITES (19, 35). WHETHER THIS LOSS EVENTUALLY PLATEAUS IS UNKNOWN BECAUSE VERY FEW HUMANS HAVE BEEN EXPOSED TO MICROGRAVITY OR TO STRICT BED REST FOR MORE THAN 6 MO. THE RODENT MODEL OF HINDLIMB UNLOADING BY TAIL SUSPENSION EFFECTIVELY MIMICS THE MICROGRAVITY ENVIRONMENT AND PRODUCES SIGNIFICANT BONE LOSS, ALLOWING FOR BASED-BASED, INVASIVE STUDIES TO BE PERFORMED (4, 22).

Crew members frequently under eat during spaceflight missions (6, 18). Food intake has been observed to be as low as 50% of a flight member’s estimated required amount and even as low as 25% in one instance (the latter assuming the adult male need 2,500 calories) (14). Reduced food intake results in decreased availability of nutrients important for maintaining bone health but may also independently affect bone status.

When these effects are combined with the reduced mechanical loading of the microgravity environment, the deleterious effect on the skeleton could be augmented.

This change of bone status due to spaceflight and/or negative energy balance involves numerous endocrine factors. Hormones such as estrogen, growth hormone, IGF-1, insulin, T₄, thyroid-stimulating hormone, calcitonin, active D₃, and parathyroid hormone have been shown to play a role in the deleterious skeletal adaptation to actual or simulated microgravity and/or to negative energy balance (23, 24, 29, 34). Leptin, a 16-kDa cytokine-like hormone principally produced by white adipocytes, may also be involved in the bone response to microgravity and/or restricted food intake. Its principal function is the regulation of energy stores and body composition through negative feedback at the hypothalamic nuclei. Leptin is now known to have numerous biological effects on the immune system, reproduction, development, hemopoiesis, angiogenesis (15, 36), and, most recently, bone metabolism.

However, there is controversy about the nature of leptin’s effects on bone. Early studies demonstrated antiosteogenic effects of leptin via the sympathetic nervous system when administered centrally (9, 33), but several more recent studies have demonstrated a bone-protective effect of leptin during hindlimb unloading or caloric restriction when administered peripherally (13, 21).

No published data, to our knowledge, test a side-by-side comparison of the effect of microgravity and/or food restriction on serum leptin levels and on bone outcomes.

Our purpose, then, was to investigate the individual and combined effects of food restriction and simulated microgravity in adult male rats and to investigate the contribution of altered serum leptin to changes in bone strength, density, and turnover status. Our primary hypotheses were that food restriction and hindlimb unloading independently impair skeletal integrity via decreased bone formation and/or increased bone resorption, resulting in diminished BMD and, if rats are subjected to both treatments, even greater decrements and changes in the above variables would result.

MATERIALS AND METHODS

Animals and Experimental Design

Forty-eight 6-month-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used in this experiment, which lasted 6 wk. The chow typically utilized by the vendor (8604 Harlan Teklad) provides excess densities of vitamins and minerals; providing 70% of baseline intake of this chow would not result in deficiencies in any key vitamins or minerals. Therefore, we chose to use for this experiment purified diet AIN93-M, a casein-based purified diet that provides...
100% of the National Research Council (NRC)-determined requirements for vitamins and minerals for laboratory rats (Table 1). Two weeks before experiment day 0, all rats (singly housed) were provided the AIN93-M diet ad libitum to acclimate to the new diet. Starting 5 days before experiment day 0, each rat’s daily intake was calculated by carefully weighing all diet pellets provided to the rat and any pellets remaining 24 h later. Each rat’s daily intake was then averaged over the 5 days to establish its baseline food requirement in grams purified diet per day. Because rats typically eat only the amount of food that they need, this calculated average was considered to be 100% of the rat’s daily food requirement.

Rats were then randomly assigned to four groups. One group (CC100) was allowed regular cage activity with each rat receiving 100% of its baseline food intake. The second group (CC70) was also allowed regular cage activity, but each rat was provided 70% of its baseline food intake. The remaining rats were subjected to hindlimb unloading by tail suspension, using a tail harness as previously described (5), and were provided 100% (HU100) or 70% (HU70) of baseline food intake. The treatment period lasted for 28 days. Once the experiment began, the CC100 and HU100 rats received the 100% amount of their individually determined daily food requirement, whereas the CC70 and HU70 rats were given 70% of their daily food requirement. Each rat’s actual food intake was verified by subtracting the weight of any diet pellets remaining in its cage from the previous day’s feeding. During the entire experiment, the rats were housed in a light-controlled room (12:12-h light-dark cycle) maintained at 21–22°C in an American Association for Accreditation of Laboratory Animal Care-accredited animal care facility. All procedures in this study were approved by the Texas A&M University Laboratory Animal Care Committee.

On day 1 of treatment and on day of death, peripheral computed tomography (pQCT) scans were performed. While the rats were anesthetized, a spot urine sample was expressed and serum was collected with a 27-gauge needle from a leg vein. For the hindlimb unloading experiment, BMD and cross-sectional geometry were measured both in vivo (tibia) on experiment days 0 and 28 and after death ex vivo (humerus and femur). Blood and urine collections were performed on both day 1 and day 28 of the treatment at the same time of the day and before 10:00 AM to minimize contribution of diurnal variation for the same variables were 0.37, 1.43, and 0.28%, respectively.

The XCT Research M (Stratec; Norland, Fort Atkinson, WI) scanner has a minimum voxel size of 0.07 mm, a scanning beam thickness of 0.50 mm, and is calibrated daily with the use of a standard hydroxyapatite phantom. In vivo scans were taken of the proximal metaphysis of the right tibia on days 0 and 28 with the animal anesthetized. Transverse images were scanned at 5.0, 5.5, and 6.0 mm from the proximal tibia plateau. Ex vivo scans were taken at the proximal humerus metaphysis (5.0, 5.5, and 6.0 mm from proximal end) and at the distal femur (6.0, 6.5, and 7.0 mm from the distal condylar edge). Bones for ex vivo scans were thawed and placed in a vial filled with 1× PBS for scanning to ensure standard hydration. A standardized analysis for either metaphyseal bone (contour mode 3, peak mode 2, outer threshold of 0.214 g/cm³, inner threshold of 0.605 g/cm³) or diaphyseal bone (separation 1, threshold of 0.605 g/cm³) was applied to each section. The same contour and peak modes and thresholds were used by our laboratory to successfully differentiate cortical and cancellous bone in skeletally mature unloaded animals (1, 4) and are explained in detail elsewhere (10). Values of total, cortical, and cancellous volumetric bone mineral density (vBMD), cross-sectional area, cortical area, and marrow area were averaged across three slices at each bone tissue to yield a mean value for each site. In addition, mid-diaphyseal cross-sectional moment of inertia was obtained with respect to the neutral bending axis during three-point bending for later calculation of material properties. Machine precision (based on manufacturer’s data) is ±3 mg/cm³ for cancellous BMD and ±9 mg/cm³ for cortical BMD. Reproducibility in our laboratory for both in vivo and ex vivo measures was determined from five repeat scans with repositioning of the animal or bone between scans. Coefficients of variation for these measurements were 1.24, 2.13, and 1.95% for in vivo proximal tibia total BMD, cancellous BMD, and total area, respectively. Ex vivo distal metaphysis coefficients of variation for the same variables were 0.37, 1.43, and 0.28%, respectively.

Biochemical Analyses

A rat osteocalcin ELISA immunoassay kit was used (Biomedical Technologies, Stoughton, MA) to measure the concentration of osteocalcin in the animals’ serum. Osteocalcin is reported as nanograms per milliliter of serum. Precision coefficients of variation within runs were ±7% and coefficients of variation between runs were ±10.5%. The concentration of urinary deoxypyridinoline (DPD) cross-links, one of the pyridinium cross-links, was assessed to estimate changes in bone resorption, using a competitive enzyme immunoassay (Pyrlinks-D; Quidel, Mountain View, CA). Results were normalized to urine creatinine, determined by a colorimetric assay (Quidel). DPD is reported as nanomole DPD per millimole creatinine. Within-run coefficient of variation was ±3.5% and between-run coefficient of variation was ±7.0%. A rat leptin ELISA immunoassay kit (Crystal Chem, Chicago, IL) was used to measure the concentration of leptin in serum and reported as nanograms per milliliter serum. The intra-assay precision coefficient of variation was ±3.1%, and the interassay run coefficient of variation was ±6.4%.

Cancellous Histomorphometry

Undemineralized distal left femora were subjected to serial dehydration and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were cut 8 μm thick and left unstained for fluorochrome label measurements and cut at 4-μm sections for von Kossa staining for measurement of cancellous bone volume normalized to tissue volume and quantification of osteoid and osteoclast surfaces as a percent of total cancellous surface.

At ×20, a defined region of interest was established ~0.8 mm from the growth plate and within the endocortical edges encompassing 8–9 mm². Total bone surface, single-labeled surface, and double-labeled surface were measured at ×100, and interlabel distances, bone volume, and osteoid/cancellous surface were measured at ×200 magnification. Mineral apposition rate (MAR; μm/day) was calculated by dividing the average interlabel width by the time between labels (7

Table 1. Comparison of feed relative to rat requirements

<table>
<thead>
<tr>
<th>Nutrient*</th>
<th>Rat Requirement1</th>
<th>AIN-93M2</th>
<th>8604 Harlan Teklad3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, g</td>
<td>50</td>
<td>125</td>
<td>244</td>
</tr>
<tr>
<td>Calcium, g</td>
<td>5</td>
<td>5</td>
<td>13.6</td>
</tr>
<tr>
<td>Magnesium, g</td>
<td>0.5</td>
<td>0.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Phosphorus, g</td>
<td>3</td>
<td>3</td>
<td>10.1</td>
</tr>
<tr>
<td>Vitamin D, IU</td>
<td>1,000</td>
<td>1,000</td>
<td>2,400</td>
</tr>
<tr>
<td>Vitamin K, mg</td>
<td>0.9</td>
<td>0.86</td>
<td>4.11</td>
</tr>
<tr>
<td>Energy, kcal/g</td>
<td>3.50</td>
<td>3.40</td>
<td></td>
</tr>
</tbody>
</table>

*Amounts of nutrients are given as unit of nutrient/kg of feed. 1As established by the National Research Council in 1995 (32). 2Purified diet fed to animals in this study (28). 3Chow diet fed by animal vendor previous to arrival at our institution.
days), and mineralizing surface (MS) for cancellous bone surfaces (BS) was calculated by using the formula MS/BS = [(single labeled surface/2) + double label surface/surface perimeter] × 100. Bone formation rate (BFR) was calculated as MAR × MS/BS. Histomorphometric analyses were performed with BioQuant True Color Windows image analysis software (BQTCW98, version 3.05.6; R&M Biometrics) interfaced with Optronics 3-chip color camera and an Olympus BX60 Microscope with epifluorescent light (Leeds Instruments, Irving, TX). All nomenclature for cancellous histomorphometry follows standard usage (25).

Statistical Analysis

To analyze pre- and posttreatment values of tibia pQCT and blood/urine variables, a three-way ANOVA with repeated measures was used. In addition, a simple main effects analysis was performed on any two-way or three-way interactions, and, when appropriate, Duncan’s post hoc tests were used within the simple main effects analyses. For end point measures (e.g., mechanical testing variables, soleus weight, and histomorphometry data), two-way ANOVA was performed, with appropriate post hoc tests. Linear associations between change of serum leptin level to the BFR were described with Pearson’s correlation coefficients. All reported values are means ± SE.

RESULTS

Food intake and body weight. Actual food intake over the 28-day experiment was low for all groups during week 1; however, during the remainder of the experiment, rats ate close to 100% of their assigned food, achieving intended food intake (Table 2). The efficacy of food restriction was demonstrated by the lack of weight gain in CC70 rats compared with control rats provided food ad libitum. By week 4, mean body weights for CC70, HU100, and HU70 groups were significantly lower than those observed in CC100 (Fig. 1). The HU100 and CC70 rats exhibited a significant drop in body weight during the first 7 days, but no further decrements were observed over the next 3 wk. The CC100 group had the highest mean body weight at death (519 ± 14 g), followed with progressively lower body weights by the HU100 group (448 ± 12 g), the CC70 group (439 ± 12 g), and then the HU70 group (388 ± 17 g), which weighed the least.

Average soleus weight results were 199.5 ± 6 g in weight-bearing CC rats and 84.5 ± 4 g in HU rats (on average, 60% lower than pooled CC value; *P < 0.0001), confirming that tail suspension effectively unloaded rats’ hindlimbs.

pQCT Data

Proximal tibial metaphysis. Hindlimb unloading and food restriction caused a reduction in total vBMD (cortical shell with cancellous core) (Fig. 2). Progressively greater reductions of total vBMD over time were observed in CC70 rats (4.7%) and HU100 rats (8.1%), with this reduction exacerbated in combined treatment (HU70) rats (9.3%). All groups exhibited equivalent significant increases in marrow area (area inside the endocortical perimeter) (Table 3). CC groups exhibited a small but nonsignificant increase in total cross-sectional area, reflecting peristaltic expansion typically observed in slowly growing adult rats (Table 3). Both HU groups exhibited no numerical change in mean total area. This lack of peristaltic expansion coupled with increased marrow area in unloaded rats resulted in reduced cortical shell area at the proximal metaphysis in these groups (Table 3), suggesting a net loss of cortical bone at this site. Cancellous vBMD at this site decreased an average of 20% in all groups over time (Table 3); there were no significant independent effects of loading condition or food restriction on this bone compartment.

Midshaft tibia and humerus. Tibial midshaft vBMD and geometry variables were not affected by HU or food restriction (Table 3). Almost all measured parameters increased over time (total area, cortical area, marrow area, cross-sectional moment of inertia) in all groups. Cortical density did not change in any group. These data suggest a uniform continued growth of midshaft cortical bone in all animals, unaffected by loading condition or food intake. No significant differences in vBMD or bone geometry were noted at the humerus midshaft with food restriction or hindlimb unloading (data not shown).

Distal Femur Cancellous Bone Histomorphometry

Because of technical error, four or five rats in each treatment group did not receive fluorochrome labels previous to death; hence, histological analyses for %MS/BS, MAR, and BFR

Table 2. Food intake

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Intake Average, g</th>
<th>70% of Baseline Intake</th>
<th>Actual Food Intake During Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Week 1*</td>
</tr>
<tr>
<td>CC100</td>
<td>23 ± 0.6</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>CC70</td>
<td>21 ± 0.6</td>
<td>16</td>
<td>17 ± 1.0</td>
</tr>
<tr>
<td>HU100</td>
<td>23 ± 0.8</td>
<td>15</td>
<td>13 ± 1.5</td>
</tr>
<tr>
<td>HU70</td>
<td>23 ± 0.9</td>
<td>15</td>
<td>17 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. Baseline intake was assessed for 5 days before actual experiment. CC100 and CC70, age-matched cage controls fed 100% and 70% of their baseline food requirements; HU100 and HU70, hindlimb unloading animals fed 100% and 70% of their baseline food requirements. *Within all groups, week 1 intake was less than that of all other weeks (P < 0.05).
were performed on femurs from 20 animals spread equally across all groups. Decreases in MAR due to food restriction (CC70) and to unloading (HU100) were similar (−25%), as were the decreases in %MS/BS for both groups (−59%) (Fig. 3). Reduction results in MAR and %MS observed in the combined treatment group (HU70) were similar to results of CC70 and HU100, which implies no additive effect of food restriction and HU on MAR and %MS/BS. These reductions in MAR and %MS/BS contributed to the 70% reduction in BFR observed in all three treatment groups. Osteoid surface was 33% lower in the CC70 and the HU100 groups than in the control group (CC100). In this case, an additive effect of food restriction and hindlimb unloading was observed; a much larger decrement of percent osteoid surface was observed in HU70 rats (60%). The bone volume normalized to tissue volume in HU rats was lower than that of CC rats by 28.5%, but this decline was not statistically significant among groups. Cancellous percent osteoclast surface did not vary among the four groups (Fig. 3, E and F).

DISCUSSION

This experiment is the first to demonstrate the independent and combined effects of food restriction and hindlimb unloading on skeletal integrity. Our data confirm our hypothesis that food restriction and hindlimb unloading independently impair skeletal integrity via decreased bone formation but probably not, as we hypothesized, by increased bone resorption. Food restriction and hindlimb unloading independently reduced total vBMD in the proximal tibia and serum leptin after 28 days. The impact of hindlimb unloading on these two variables was significantly greater than that of food restriction, and some additive effects of the combined treatment were also observed. Reduction in total vBMD appears to be because of a thinner cortical shell after hindlimb unloading at this metaphyseal site.

Leptin and Turnover Markers

Compared with results for day 0, decreases in serum leptin levels (Fig. 4A) were observed by 28 days in all groups except CC100, with 27% and 60% reductions observed in CC70 and HU100 groups, respectively. Serum leptin was not detectable after 28 days in HU70 rats. Assays for this group were repeated with increasing serum volumes (5, 10, and 20 μl), but serum leptin remained undetectable. The change in serum leptin level over the experimental period correlated well with BFR. Those rats exhibiting the greatest decline in serum leptin had the lowest BFRs in distal femur cancellous bone (Fig. 4B).

There were significant main effects of food restriction and of hindlimb unloading on serum osteocalcin. The mean serum osteocalcin for the food-restricted rats was 32% lower at the end of the experimental period than that of all 100%-fed rats, whereas mean osteocalcin values for all HU animals exhibited a greater decline in serum osteocalcin (−23%) vs. that observed in all CC animals (−10%) (P < 0.01). Mean urine DPD, the resorption marker, increased in all groups over the experimental period, rising by 17% (CC100), 24% (CC70), 29% (HU100), and 46% (HU70) over 28 days (data not shown). However, the magnitudes of increase in DPD were not statistically different among groups.

Table 3. In vivo peripheral computed tomography measures of food restriction and/or HU effects on tibia density and geometry

<table>
<thead>
<tr>
<th></th>
<th>CC100</th>
<th>CC70</th>
<th>HU100</th>
<th>HU70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 Day 28</td>
<td>Day 0 Day 28</td>
<td>Day 0 Day 28</td>
<td>Day 0 Day 28</td>
</tr>
<tr>
<td>Proximal tibia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancellous vBMD, mg/cm³</td>
<td>203±7 166±6</td>
<td>204±7 167±5</td>
<td>214±7 161±5</td>
<td>221±8 180±5</td>
</tr>
<tr>
<td>Marrow area, cm²</td>
<td>10.3±0.5 10.8±0.7</td>
<td>10.0±0.4 10.5±0.5</td>
<td>11.3±0.5 11.8±0.5</td>
<td>10.5±0.5 11.2±0.6</td>
</tr>
<tr>
<td>Total area, cm²</td>
<td>19.0±0.5 19.5±0.8</td>
<td>18.5±0.6 18.9±0.6</td>
<td>20.1±0.7 20.1±0.6</td>
<td>19.1±0.8 19.1±0.8</td>
</tr>
<tr>
<td>Cortical shell area, cm²</td>
<td>8.7±0.2 8.8±0.2</td>
<td>8.5±0.2 8.4±0.2</td>
<td>8.8±0.2 8.3±0.2§</td>
<td>8.6±0.3 7.9±0.3§</td>
</tr>
<tr>
<td>Tibia diaphysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical vBMD, mg/cm³</td>
<td>1.324±7 1.326±7</td>
<td>1.324±7 1.317±15</td>
<td>1.312±6 1.328±6</td>
<td>1.315±4 1.329±2</td>
</tr>
<tr>
<td>Cortical area, cm²</td>
<td>6.2±0.2 6.2±0.2</td>
<td>6.2±0.1 6.2±0.2</td>
<td>6.2±0.1 6.3±0.1</td>
<td>6.0±0.1 6.1±0.1</td>
</tr>
<tr>
<td>Total area, cm²</td>
<td>8.4±0.3 8.6±0.3</td>
<td>8.5±0.2 8.7±0.3</td>
<td>8.9±0.2 9.0±0.2</td>
<td>8.4±0.2 8.5±0.2</td>
</tr>
<tr>
<td>Marrow area, cm²</td>
<td>2.3±0.1 2.3±0.2</td>
<td>2.3±0.1 2.4±0.1</td>
<td>2.6±0.1 2.7±0.1</td>
<td>2.3±0.1 2.4±0.1</td>
</tr>
<tr>
<td>CSMI, mm⁴</td>
<td>11.2±0.7 12.3±0.8</td>
<td>11.3±0.5 12.1±0.7</td>
<td>12.1±0.4 12.8±0.4</td>
<td>11.0±0.6 11.5±0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. vBMD, volumetric bone mineral density; CSMI, cross-sectional moment of inertia. For all experimental groups, n = 11, except for HU100, in which n = 12. All groups' means increased over 28 days vs. day 0 (P < 0.01). All groups' means decreased over 28 days vs. day 0 (P < 0.0001).§All groups' values increased over time (P ≤ 0.005). The HU group's value decreased over time more than that of the CC group (P < 0.05).
although all groups also incurred some loss of cancellous vBMD.

These declines in metaphyseal bone mass can also be attributed to consistent decreases in MAR and especially MS/BS, leading to significant reductions in BFR in cancellous bone observed in both food restriction and HU rats. Osteoid surface was the only histomorphometric variable that showed an additive effect when both treatments were applied. Osteoid deposition is an early step in formation of new bone, which must precede mineralization. This additive effect of restricted food intake and hindlimb unloading was not observed in the mineralization-dependent variables after 28 days. It is reasonable to surmise that a longer duration of unloading combined with food restriction would eventually yield greater decrements in mineralization and BFRs.

We could not confirm an additive effect of unloading and food restriction on proximal tibial cancellous vBMD in this study. Proximal tibial cancellous vBMD after 28 days was not significantly different among the four groups; a significant 18% reduction in cancellous vBMD was observed even in weight-bearing control (CC100) rats. This decline in cancellous vBMD across all groups is consistent with the elevation in urinary DPD observed in all groups, although increases in systemic resorption markers cannot pinpoint the bone sites experiencing increased resorption. It is important to note that all rats in the present study were switched from 8604 Harlan Teklad rodent diet (fed by vendor until rats were supplied at 5 mo of age) to AIN93-M diet 5 days before the experiment started. The 8604 chow has a high content of minerals and vitamins, providing about three times the nutrient requirement for laboratory rats established by the NRC (32). In this study, the use of a purified diet containing the minimum amount of nutrients for good rodent health was required to effectively restrict nutrient intake below those of NRC-recommended intakes. Although purified diet AIN93-M meets 100% of rats’ nutrient and energy requirements, a switch to AIN93-M at the start of the experiment from the 8604 chow fed by the vendor did result in reduced vitamin, mineral, and protein intake.

Dietary protein restriction lowers plasma IGF-I, impairs cortical bone formation, and induces osteoblastic resistance to...
unloading and no differences in percent osteoclast surface among the groups, as measured at the distal femur. As a whole body measure, biochemical markers of bone turnover showed more responsive changes in a marker of bone formation (osteocalcin) to imposed nutritional and/or mechanical environment changes than in a marker of bone resorption (DPD). Decreases in osteocalcin concentration and bone formation activity in young rapidly growing rats have been measured (20). Serum osteocalcin transiently decreases by 25% in 6-wk-old rats after 1 wk of hindlimb unloading and then returns to almost normal levels after 28 days of hindlimb unloading (26). However, there are few published data on the osteocalcin responses to hindlimb unloading in adult rats. The skeletally mature (6-mo-old) male rats used in the present study subjected to hindlimb unloading experienced an average decline of 23% osteocalcin measured after 28 days. To our knowledge, only one published study reports urinary DPD measurements on tail-suspended rats, which has exhibited the same trends as our study (27). By contrast, consistent and dramatic increases in DPD have been demonstrated in humans exposed to space missions lasting 4 – 6 mo. In crew members on space missions for 4 – 6 mo, DPD increased 55% above preflight levels (30). It may well be that underlying tissue mechanisms (decreased formation vs. increased resorption) for disuse bone loss in rodents vary from those in humans.

It has been proposed that leptin is responsible for linking energy metabolism to bone mass and may also play a role in bone loss during hindlimb unloading. To our knowledge, our study’s results provide the first documentation of some additive effects of microgravity and food restriction on serum leptin and skeletal integrity. In the present study, we found a significant decrease in serum leptin after 28 days in response to hindlimb unloading and to food restriction, with undetectable levels of serum leptin in rats exposed to both treatments, suggesting a striking effect of the combined treatments on serum leptin levels. The decrement in serum leptin was strongly associated with the decline in BFR. This finding is consistent with previous studies that demonstrated a 40 – 60% decrease in leptin observed with hindlimb unloading in rats and a positive effect of leptin replacement on bone (2, 21). The decrease in leptin with this hindlimb unloading model is also of great interest because a similar decrease has been observed in human subjects during spaceflight (31). However, there still exists some controversy about the putative effects of leptin on bone. Evidence for a central (central nervous system) action for leptin has been provided by Takeda et al. (33), who demonstrated that leptin’s anorexigenic and antiosteogenic effects act via two distinct neuronal pathways involving the sympathetic nervous system.

One possible mechanism for the decrease in serum leptin with food restriction or hindlimb unloading may be the attendant reduction of fat mass, which is the main source of leptin secretion. Our laboratory observed a mean 38% decrement in total body fat mass with 4 wk of 40% energy restriction in female rats and a mean 8% decrement in total body fat mass with 4 wk of hindlimb unloading in male rats (unpublished data). In the present study, very interestingly, 28 days of hindlimb unloading had a greater suppressive effect on serum leptin than did restricting food intake. Given that rats exposed only to unloading had lower serum leptin but higher body weights than rats exposed to only food restriction by the end of

IGF-I in adult female rats (7). An uncoupling of bone resorption and formation is observed in adult male rats maintained on an isocaloric low-protein diet (8). Dietary calcium restriction results in reduced BMD and cross-sectional moment of inertia in femoral and vertebral bone; it also impairs normal bone remodeling, uncoupling bone formation from bone resorption (16). The reductions in dietary intake of protein and calcium imposed at the beginning of this study with the switch to the AIN93-M diet may explain the decrement in cancellous vBMD observed in control animals (CC100); we speculate that this represents an adaptive downregulation of cancellous bone mass to reduced availability of calcium and perhaps also protein.

Recent studies have demonstrated that 40% food restriction causes a significant increase in disuse-like bone turnover of endocortical bone in the tibial diaphysis of 13-mo-old female rats (3) and that 20% food restriction augments decrements in bone mineral content and vBMD in total tibia of 7-mo-old ovariectomized rats (11), supporting our findings. Ideally, a more prolonged acclimatization period for rats to new chow should be utilized to allow changes in vBMD and other key variables to plateau.

In the bone microenvironment, there is a dynamic balance between resorption and formation that maintains skeletal homeostasis. In the present study, we demonstrated reduced percent osteoid surface with food restriction and/or hindlimb

![Fig. 4. A: serum leptin levels after 28 days of food restriction or HU in rats consuming 100% or 70% of usual food intake. All groups are n = 11 except for HU100, in which n = 12. ND, not detectable. *P < 0.0001 vs. prevalue; #P < 0.05 vs. postvalue in CC70 group. B: correlation between change in serum leptin value and BFR measured at 28 days for 20 animals.](http://jap.physiology.org/)

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**Fig. 4.** A: serum leptin levels after 28 days of food restriction or HU in rats consuming 100% or 70% of usual food intake. All groups are n = 11 except for HU100, in which n = 12. ND, not detectable. *P < 0.0001 vs. prevalue; #P < 0.05 vs. postvalue in CC70 group. B: correlation between change in serum leptin value and BFR measured at 28 days for 20 animals.
the experiment, it may be that some factor other than body weight and food intake is associated with regulation of serum leptin. We speculate that increased stress or increased sympathetic neural output may be another potential regulator of serum leptin levels, which then may impact on bone outcomes (12, 17, 37). In future studies that use food restriction and hindlimb unloading, it would be useful to assess changes in sympathetic function (e.g., urinary corticosterone). IGF-I is another key hormonal marker of energy balance, and alterations in this endocrine regulator of osteoblast activity could be another contributor to the bone loss observed in this study.

In summary, moderate food restriction caused nearly as much bone loss at the proximal tibia as did the unloading effect of simulated microgravity. This appears to be due primarily to suppressed osteoblast activity in response to changes in both the nutritional and mechanical environment. We could not detect consistent changes in indicators of bone resorption. We conclude that bone loss during hindlimb unloading is greater than with food restriction and were associated with declines in BFR. These data provide evidence for an additional negative effect of undernutrition in the context of disuse bone loss. We conclude that bone loss during spaceflight could be aggravated by consistent reductions in food intake as is frequently observed during short-term shuttle missions. These results may also have serious implications for bed-rest patients who restrict food intake or for those individuals who utilize food restriction in attempts to lose weight. Serum leptin may be an important endocrine regulator contributing to this change in bone metabolism.

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