Effect of gender on bone turnover in adult rats during simulated weightlessness

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Hefferan, T. E., G. L. Evans, S. Lotinun, M. Zhang, E. Morey-Holton, and R. T. Turner. Effect of gender on bone turnover in adult rats during simulated weightlessness. J Appl Physiol 95: 1775–1780, 2003. First published July 25, 2003; 10.1152/japplphysiol.00455.2002.—Prolonged space-flight results in bone loss in astronauts, but there is considerable individual variation. The goal of this rat study was to determine whether gender influences bone loss during simulated weightlessness. Six-month-old Fisher 344 rats were hindlimb unweighted for 2 wk, after which the proximal tibiae were evaluated by histomorphometry. There were gender differences in tibia length, bone area, cancellous bone architecture, and bone formation. Compared with female rats, male rats had an 11.6% longer tibia, a 27.8% greater cortical bone area, and a 37.6% greater trabecular separation. Conversely, female rats had greater cortical (316%) and cancellous (145%) bone formation rates, 28.6% more cancellous bone, and 30% greater trabecular number. Hindlimb unweighting resulted in large reductions in periosteal bone formation and mineral apposition rate in both genders. Unweighting also caused cancellous bone loss in both genders; trabecular number was decreased, and trabecular separation was increased. There was, however, no change in trabecular thickness in either gender. These architectural changes in cancellous bone were associated with decreases in bone formation and steady-state mRNA levels for bone matrix proteins and cancellous bone resorption. In conclusion, there are major gender-related differences in bone mass and turnover; however, the bone loss in hindlimb unweighted adult male and female rats appears to be due to similar mechanisms.

Changes in bone resorption have not been detected in the growing male rat during spaceflight (7). However, when pregnant rats were flown in space, increased osteoclast number was observed after flight (33). In addition, spaceflight resulted in an increase in cancellous bone loss in ovariectomized rats over and above that caused by ovarian hormone deficiency. Bone formation was not reduced in these animals, implying that the bone loss was entirely due to increased bone resorption (8). Similar results were observed in the skeleton of hindlimb-unloaded ovariectomized rats (26). These findings suggest that there may be a gender-dependent skeletal response to unweighting. Such differences, were they to occur, could complicate the effective application of countermeasures to prevent spaceflight-induced bone loss.

There is, however, an alternative explanation. It may be more difficult to detect an increase in bone resorption in rapidly growing male rats than comparably aged females, which are smaller and grow more slowly (8, 16, 21). Thus it is possible that the observed differences between male and female rats are related to skeletal maturity and gonadal status rather than to gender. To identify potential gender differences, we subjected adult male and female rats to hindlimb unloading, a well-established ground-based model for simulating weightlessness (20).

MATERIALS AND METHODS

Animals. The animal protocol was approved by the institutional animal welfare committees at the Mayo Foundation (Rochester, MN) and National Aeronautics and Space Administration (NASA)-Ames Research Center (Moffett Field, CA).

Intact, virgin, 6-mo-old female and male Fisher 344 rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained on a 12-h light-dark cycle. The animals were housed in individual cages in gender-specific rooms, because cohabitation increases stress. The baseline groups consisted of baseline female (n = 8) and baseline male (n = 7) rats. The weight-bearing control groups consisted of female (n = 10) and male (n = 10) rats, and the hindlimb-unloaded groups consisted of female (n = 12) and male (n = 12) rats. The baseline and hindlimb-unloaded groups were fed standard lab chow and water ad libitum, whereas control animals...
were pair fed to the appropriate hindlimb-unloaded group to control for caloric intake. The hindlimb-unloaded groups were unloaded by using the Morey-Holton protocol (20) and housed in specially designed metabolic cages (14).

To assess bone formation and resorption, animals were labeled with fluorochromes by subcutaneous injections. All animals received a baseline label on day 0 of the experiment (20 mg/kg oxytetracycline; Sigma Chemical, St. Louis, MO). The baseline groups were killed 24 h later on day 1. The unloaded groups were unloaded on day 1. The second fluorochrome label, 20 mg/kg calcein (Sigma Chemical), was administered 8 days before death, and the third fluorochrome label, 20 mg/kg tetracycline (Sigma Chemical), was given 2 days before death. At the end of 14 days of unloading, the animals were anesthetized by CO2 and decapitated. The uterus and seminal vesicles were weighed. The right tibia was fixed in 70% ethanol, and length was measured by using a precision caliper and processed for histomorphometry.

Bone histomorphometry. The histomorphometric measurements were performed by using the OsteoMeasure Analysis System (Osteometrics, Atlanta, GA), as has been previously described (8).

Cortical bone histomorphometry. Ground sections were cut proximal to the tibia-fibula synostosis and prepared as described (26). The following measurements and calculated values were obtained as described (26): cross-sectional area (CSA), medullary area (MA), periosteal bone area (CA), periosteal double-labeled surface (dLS), periosteal mineral apposition rate (MAR), periosteal bone formation rate (PsBFR), and periosteal mineral apposition rate (PsMAR). Post hoc comparisons were made by using Fisher’s protected least significant difference test, with statistical significance defined as \( P < 0.05 \). In addition, the respective effects of gender and hindlimb unloading were determined by two-way ANOVA by using SuperANOVA software (Abacus Concepts). The following static bone measurements and calculated values were as described (26): bone volume normalized to tissue volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), and trabecular separation (TbSp). The following dynamic bone measurements and calculated values were obtained as described (26): baseline label perimeter, dLS, MAR, and BFR normalized to bone surface. Resorption was calculated as the difference in oxytetracycline-labeled surface between the baseline and either the control or unweighted groups (8, 26).

Table 1. Effects of gender and hindlimb unloading on body, uterine, and seminal vesicle weights and tibia length

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Female Rats</th>
<th>Male Rats</th>
<th>Two-way ANOVA, P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline*</td>
<td>Control</td>
<td>Unloaded</td>
</tr>
<tr>
<td>Starting weight, g</td>
<td>192 ± 3</td>
<td>193 ± 4</td>
<td>194 ± 3</td>
</tr>
<tr>
<td>Final weight, g</td>
<td>194 ± 0.3</td>
<td>182 ± 0.5</td>
<td>170 ± 0.4‡</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine weight, g</td>
<td>0.81 ± 0.07</td>
<td>0.43 ± 0.03*</td>
<td>0.40 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tibia length, cm</td>
<td>3.61 ± 0.02</td>
<td>3.68 ± 0.02</td>
<td>3.62 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. NA, not applicable; NS, not significant. *No significant difference compared to baseline values were observed. ‡\( P < 0.01 \) compared with female control. †\( P < 0.05 \) compared with control.

Table 2. Effects of gender and hindlimb unloading on cortical bone histomorphometry

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Female Rats</th>
<th>Male Rats</th>
<th>Two-way ANOVA, P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline*</td>
<td>Control</td>
<td>Unloaded</td>
</tr>
<tr>
<td>CSA, mm²</td>
<td>3.46 ± 0.06</td>
<td>3.46 ± 0.04</td>
<td>3.43 ± 0.05</td>
</tr>
<tr>
<td>CA, mm²</td>
<td>2.85 ± 0.04</td>
<td>2.85 ± 0.03</td>
<td>2.79 ± 0.04</td>
</tr>
<tr>
<td>MA, mm²</td>
<td>1.62 ± 0.02</td>
<td>0.61 ± 0.01</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>dLS, mm</td>
<td>1.15 ± 0.01</td>
<td>0.12 ± 0.01*</td>
<td>0.18 ± 0.07‡</td>
</tr>
<tr>
<td>PsBFR, mm²/10⁻³/day</td>
<td>2.41 ± 0.42</td>
<td>0.27 ± 0.10‡</td>
<td>0.58 ± 0.15</td>
</tr>
<tr>
<td>PsMAR, μm/day</td>
<td>1.98 ± 0.21</td>
<td>0.68 ± 0.26*</td>
<td>1.22 ± 0.25</td>
</tr>
</tbody>
</table>

Values are means ± SE. CA, cortical area; CSA, cross-sectional area; MA, medullary area; PsBFR, periosteal bone formation rate; PsMAR, periosteal mineral apposition rate; dLS, double-label surface. *No significant difference compared to baseline values were observed. ‡\( P < 0.01 \) compared with female control. †\( P < 0.05 \) compared with control.
male and female rats in the treatment groups were significantly decreased compared with their respective baseline controls. These differences were partly (46% female, 82% male) due to the reduced caloric intake of the hindlimb-unweighted animals since pair feeding the control group to the unweighted group resulted in decreases in body weight. The weight loss in female rats (−24 g; 12%) was much less than in male rats (−67 g; 20%). Pair feeding resulted in an overall average 25% reduction in food intake compared with age-matched animals fed ad libitum, but there was no significant difference in daily food consumption between the pair-fed control and the unweighted groups. Male rats had longer tibia than female rats. Hindlimb unweighting had no effect on tibia length. Uterine and seminal vesicle weights were significantly increased in the hindlimb-unweighted group compared with the respective baseline group. The declines in uterine and seminal vesicle weights were associated with nutrition rather than unloading, because similar decreases occurred in the pair-fed weight-bearing controls.

The cortical bone histomorphometry is summarized in Table 2. There were gender differences in all static and dynamic bone measurements. Male rats had the larger values for the static measurements (CA, CSA, and MA), and the female rats had larger values for the dynamic measurements (dLS, MAR, and BFR). There were no significant effects of hindlimb unweighting on the static bone measurements. Unweighting resulted in a significant reduction in the periosteal BFR in both genders. This large reduction in bone formation was due to significant decreases in dLS and MAR.

Cancellous bone architecture is shown in Table 3. BV/TV was larger in female rats as was TbN. There was no gender difference in TbTh, but TbSp was larger in male rats. Hindlimb unweighting resulted in similar changes in bone architecture in both genders. There were decreases in BV/TV and TbN. No changes in TbTh were observed, and TbSp was increased.

The effects of gender and hindlimb unweighting on dynamic cancellous bone measurements are shown in Figs. 1–4. There were gender differences for all measurements; weight-bearing female rats had more dLS (Fig. 1), a greater MAR (Fig. 2), a greater BFR (Fig. 3), and more pretreatment single-label surface (Fig. 4) than male rats. Hindlimb unweighting resulted in significant decreases in dLS, MAR, BFR/BS, and pretreatment single-label surface in both genders.

The expression of bone matrix genes is shown in Table 4. Type I collagen was decreased 23% in the female unweighted group and 25% in the male unweighted group compared with the respective controls, whereas osteonectin was decreased 31% in the female rats and 42% in the male rats. Osteocalcin steady-state mRNA expression was decreased with unweighting 36 and 43% in both the female and the male rats, respectively. Overall, the expression of the bone matrix protein steady-state mRNA was higher in the female rats than in the male rats.

### Table 2. Gender differences in bone measurements in 6-month-old rats

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Female Rats</th>
<th>Male Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline*</td>
<td>Control</td>
<td>Unloaded</td>
</tr>
<tr>
<td>Baseline*</td>
<td>Control</td>
<td>Unloaded</td>
</tr>
<tr>
<td>Gender</td>
<td>Unloaded</td>
<td>Interaction</td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>26.8±3.2</td>
<td>25.1±0.9</td>
</tr>
<tr>
<td>TbTh, μm</td>
<td>68.1±3.6</td>
<td>68.4±1.3</td>
</tr>
<tr>
<td>TbN, mm⁻¹</td>
<td>3.9±0.1</td>
<td>3.7±0.1</td>
</tr>
<tr>
<td>TbSp, μm</td>
<td>190±12</td>
<td>206±9</td>
</tr>
</tbody>
</table>

Values are means ± SE. BV, bone volume; TV, tissue volume; TbTh, trabecular thickness; TbN, trabecular number; TbSp, trabecular separation. *No significant differences compared with baseline values were observed. †P < 0.01 compared with male control.
Changes in bone matrix proteins: Table 4

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Female Rats</th>
<th>Male Rats</th>
<th>Two-way ANOVA, P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Unloaded</td>
<td>Control</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>0.52 ± 0.06</td>
<td>0.12 ± 0.06*</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>0.75 ± 0.09</td>
<td>0.23 ± 0.05*</td>
<td>0.38 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6) of matrix protein mRNA/18S rRNA levels. *P < 0.01 compared with female control. †P < 0.01 compared with male control.

DISCUSSION

As is the case in many species of vertebrates, there is pronounced gender difference in bone mass in rats where sexually mature males have longer, more robust long bones than comparable-age females (29). In this study, we have shown that histomorphometric examination of the proximal tibial metaphysis reveals that female rats have a higher cancellous bone volume (normalized to tissue volume) than male rats. The more abundant cancellous bone mass in female rats can be attributed to estrogen. Secretion of estrogen at puberty reduces resorption of calcified growth plate cartilage during endochondral ossification (5, 18) by inhibiting the fusion of chondroblasts from their circulating precursors (27). The volume of primary spongiosa is thus increased without an increase in longitudinal bone growth (27). Increased retention of primary spongiosa provides a more extensive template for deposition of bone matrix by differentiating osteoblasts, leading to an increase in cancellous bone volume without an accompanying increase in BFR (perimeter referent) (27).

The unanticipated gender difference in the periosteal BFR, where female rats had a higher BFR but lower cortical bone mass, may be related to the inhibitory effect of caloric restriction on male rats. The unweighted male rats and their pair-fed controls ate less food and lost more weight than the comparable female rats. Caloric restriction has been shown to be a potent inhibitor of radial bone growth (30).

The greater cancellous bone volume in female rats was associated with differences in bone architecture. Trabeculae were more closely spaced than in male rats, but there was no gender difference in TbTh. The higher BFR in female rats was due to an increase in mineralizing surface; there was no difference in MAR.

Unloading hindlimbs had dramatic inhibitory effects on periosteal bone formation in both genders. Thus the lack of an accompanying change in the static measurements is attributable to the short duration of the study and slow radial growth in 6-mo-old rats. The inhibition in bone formation in hindlimb-unweighted limbs of both genders was due a combination of decreased dLS/BS and decreased MAR.

Hindlimb unweighting resulted in gender-independence decreases in cancellous bone volume (BV/TV) compared with baseline values. This bone loss was accompanied by architectural changes; there were decreases in TbN with corresponding increases in TbSp. Decreases in BV/TV in both genders were associated with decreased bone formation and increased bone resorption. Bone resorption was calculated as the difference in baseline and treatment oxytetracycline-labeled surface; therefore, a greater loss of pretreatment label indicates increased resorption. These results differ from growing male rats in which osteopenia was due to decreased bone formation only (12, 17, 25, 28, 32, 43) and ovariectomized rats in which osteopenia was due to increased bone resorption only (8, 37). Analysis of biochemical markers for bone metabolism suggests that the bone loss in astronauts during spaceflight is likely due to a combination of decreased bone formation.
tion and increased bone resorption (1, 6, 15, 22, 38, 39). However, bone histology has not been investigated in astronauts, and the changes in biochemical markers of bone metabolism have not been entirely consistent (24).

There were decreases in uterine weight and seminal vesicle weights in hindlimb-unloaded female and male rats compared with their respective baseline controls, suggesting a reduction in circulating levels of sex hormones. Decreased testosterone levels have been reported in rats during spaceflight and after hindlimb unloading of growing rats (31, 40), and in astronauts (23). In the present study, however, serum testosterone and 17β-estradiol was not reduced by unweighting in male rats, respectively (data not shown). There was no difference in uterine and seminal vesicle weights between the hindlimb-unweighted and paired controls, indicating that the decreases in reproductive organ weight in male and female rats were due to caloric restriction and not to unloading per se.

Our results using simulated weightlessness suggest that gender does not determine the skeletal response to unloading in normal adult rats. On the other hand, estrogen can influence the response. In ovariectomized rats, both BFR and bone resorption rate are increased due to severe estrogen deficiency. Neither hindlimb unloading nor spaceflight reduced cancellous bone formation in ovariectomized rats (8, 37). The excess bone loss in ovariectomized animals, in contrast to unweighted male and ovariectomy intact female rats, was entirely due to increased bone resorption. Additionally, administration of estrogen agonists reduces bone loss in male rats subjected to sciatic neurectomy, a more drastic disuse model (35), as well as female rats (Turner, unpublished observations) by suppressing bone turnover. Finally, there is evidence that mechanical signaling acts on bone cells, in part, through estrogen receptors (10). It is not yet clear whether androgens have similar actions.

In summary, hindlimb unweighting, an Earth-based model for spaceflight, results in cancellous bone loss in adults rats because of uncoupled bone turnover; bone resorption is increased, but bone formation is decreased. Similar changes occur in male and female rats, suggesting similar mechanisms of action. Further long-term studies are required to determine whether gender influences either the magnitude of the bone loss or extent of recovery after reestablishment of normal weight bearing.

The authors acknowledge Lori Rolbiecki for secretarial assistance and Peggy Backup for editorial assistance.

DISCLOSURES

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