Original Article

Differential effect of short-term etidronate treatment on three cancellous bone sites in orchidectomized adult rats

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Abstract. The aim of the present study was to analyze the effects of short-term treatment with the antiresorptive agent, etidronate, on orchidectomized adult rats, via comparison of three cancellous bone sites, the lumbar vertebral body (LVB), proximal tibial metaphysis (PTM), and distal tibial metaphysis (DTM). Thirty-five male Wistar rats, aged 10 months, were randomly divided into four groups: baseline control (BLC, n = 10), age-matched sham-operated control (AMC, n = 9), orchidectomy (ORX, n = 9), and ORX + etidronate treatment (n = 7). Etidronate treatment (10 mg/kg, daily subcutaneous injection) was initiated 2 weeks after surgery and was continued for 2 weeks. Four weeks after surgery, the 5th LVB, PTM, and DTM were processed for histomorphometric analysis of cancellous bone (secondary spongiosa). ORX resulted in a decrease in body weight. No significant difference in cancellous bone volume (BV/TV) was found between the BLC and AMC groups at any skeletal site. The cancellous BV/TV loss was attributable to increased eroded surface (ES/BS) with no significant alteration in the mineral apposition rate (MAR), at all skeletal sites and etidronate treatment in ORX rats significantly decreased ES/BS to a level not significantly different from that in the AMC group, resulting in complete prevention of ORX-induced cancellous BV/TV loss. The MAR was markedly decreased in the PTM and LVB, but maintained in the DTM by etidronate treatment. The present study showed that etidronate treatment could completely prevent ORX-induced cancellous bone loss regardless skeletal sites by suppressing bone resorption. In particular, suppression of bone formation in terms of osteoblastic activity by etidronate treatment was not evident only in the DTM.


Key words: orchidectomy, etidronate, bone histomorphometry, distal tibial metaphysis (DTM), adult rats

Introduction

The orchidectomized rat model has been widely accepted for studying the prevention of androgen-deficient cancellous bone loss.1–5 Testosterone deficiency induced by orchidectomy (ORX) inhibits cancellous bone gain in rapidly growing male rats, and induces cancellous osteopenia in adult rats as a result of increased bone turnover.6–8 Most of the studies have focused on the proximal tibial metaphysis (PTM), distal femoral metaphysis, and lumbar vertebral body (LVB), which are sites with a nonfused growth plate.

To date, it has been reported that ovariectomy in 3-month-old rats does not induce cancellous bone loss in the distal tibial metaphysis (DTM) with no alteration in bone formation endpoints, and treating ovariectomized rats with prostaglandin-E2 prevents age-related bone changes, adds extra bone, and improves the microanatomical structure by stimulating bone formation without altering bone resorption.9 It has also been reported that prostaglandin-E2 treatment induces more bone formation in the DTM than in the PTM in adult rats.10 The DTM has a fully closed growth plate in adult rats because the growth plate closes at 3 months.11

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Thus, the cancellous bone response to treatment with an anabolic agent, prostaglandin-E$_2$ in the DTM has been considered greater than that in the PTM in adult rats. However, very few studies have reported the alteration of cancellous bone in the DTM of orchidectomized adult rats, and the comparative response of cancellous bone in the DTM and PTM to antiresorptive agents has rarely been reported, although it was reported that etidronate treatment prevented loss of bone mineral density in the proximal tibia, distal femur, and LVB in ORX rats.$^{12}$ The aim of the present study was to compare the response of cancellous bone to ORX and short-term etidronate treatment for ORX-induced cancellous osteopenia among the three skeletal sites, the LVB, PTM, and DTM in adult rats.

Materials and Methods

Treatment of animals

Forty 4-week-old male Wistar rats were purchased from SLC Japan Inc. (Tokyo, Japan) and acclimatized for 9 months under standard laboratory conditions at a room temperature of 23 $\pm$ 2°C and a humidity of 55 $\pm$ 5%. The rats were allowed free access to tap water and commercial standard rodent chow (CE-2: Clea Japan Inc., Tokyo, Japan) containing 1.25% calcium, 1.06% phosphorus and 2.0 IU/g vitamin D$_3$.

Rats aged 10 months were used. Ten rats served as baseline control (BLC). The remaining 30 rats underwent ORX or sham-operation under ether anesthesia, and were randomly divided into three groups of 10 rats each: age-matched sham-operated control (AMC), ORX and ORX + etidronate treatment (E). Body weight was measured every day. Bisphosphonate etidronate was synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). Etidronate treatment (10 mg/kg body weight, daily subcutaneous injection) was initiated 2 weeks after surgery when the surgical wound was completely healed, and was continued for 2 weeks. The dose of etidronate was determined based on the results of a previous study.$^{13}$ During the experiment, one rat in each of the AMC and ORX groups and three rats in the ORX + E group were removed from the study because they spontaneously died within several days postsurgery probably as a complication of surgery. Animal breeding was performed at Sumitomo Pharmaceuticals Research Center, Osaka, Japan. These animal studies were carried out in accordance with Sumitomo Pharmaceuticals’ ethical guidelines for animal care.

Preparation of specimens

Rats in the BLC group were killed by exsanguination under ether anesthesia at the beginning of the experiment. Rats in the AMC, ORX, and ORX + E groups were labeled by subcutaneous injection of 8 mg/kg calcine and 20 mg/kg tetracycline at 14 days and 7 days before killing, respectively. They were killed by exsanguination under ether anesthesia at 4 weeks after surgery. The left tibia and 5$^{10}$ LVB were removed and stored in 70% ethanol at 4°C. The tibia was cut using a diamond band saw and divided into three parts: the proximal tibia, middle tibia, and distal tibia. The LVB was also isolated using a diamond band saw. The PTM, DTM, and LVB were fixed in 70% ethanol and stained with an anabolic agent, prostaglandin-E$_2$ in the DTM.

Bone histomorphometry

The image of the specimen, observed under a fluorescence microscope and recorded with a video camera (DK-3000, Hitachi, Japan), was processed using a plotter (Cosmozone 1SA, Nikon, Japan). The following primary parameters for cancellous bone were measured: total tissue volume (TV, $\mu$m$^3$), bone volume (BV, $\mu$m$^3$), bone surface (BS, $\mu$m), eroded surface (ES, $\mu$m), single-labeled surface (SLS, $\mu$m), double-labeled surface (dLS, $\mu$m), and interlabel width (IrLWi, $\mu$m). From these primary parameters, the following parameters were calculated according to the standard nomenclature described by Parfitt, et al.$^{15}$: bone volume (BV/TV, %), eroded surface (ES/BS, %), mineralizing surface (MS/BS, %), mineral apposition rate (MAR, $\mu$m/day), bone formation rate/BS (BFR/BS, $\mu$m$^3$/ $\mu$m$^2$/year), and BFR/BV (%/year). In the present study, the region of cancellous bone measured was 1–3 mm distal to the growth plate for the PTM, 0–2 mm proximal to the closed growth plate for the DTM, and 1–3 mm cranial to the caudal growth plate for the LVB. All of those areas corresponded to the secondary spongiosa.

Statistical analysis

All data in the tables and figures are presented as mean $\pm$ standard deviation (SD). The changes in body weight with time in the AMC group were evaluated by one-way analysis of variance (ANOVA) with repeated measurements. The changes in body weight with time were compared between the AMC and other groups using two-way ANOVA with repeated measurements. Multiple comparisons of data such as body weight at each time point and bone histomorphometric parame-
Comparisons among the groups were performed by ANOVA with Fisher’s protected least significant difference (PLSD) test. P < 0.05 was considered statistically significant. All statistical analyses were performed using the Stat View J-5.0 program on a Macintosh computer.

Results

Body weight

Table 1 shows the changes in body weight. Baseline body weight did not differ significantly among the groups. The longitudinal changes in body weight in the AMC group were not significant (one-way ANOVA). Body weight in the ORX and ORX + E groups significantly decreased longitudinally compared with the AMC group (both P < 0.05, two-way ANOVA).

Bone histomorphometry

Figure 1 and Tables 2, 3, and 4 show the structural variables and the formative and resorptive variables of cancellous bone in the PTM, DTM, and 5th LVB. No significant difference in cancellous BV/TV was found between the BLC and AMC groups at any skeletal site. Bone turnover, as indicated by BFR/BV in the AMC group, was lower in the DTM than in the PTM and LVB and lower in the LVB than in the PTM. However, the magnitude of ORX-induced cancellous BV/TV loss greater in the PTM and DTM than in the LVB and greater in the DTM than in the PTM. The cancellous BV/TV loss was attributable to increased ES/BS with increased MS/BS and no significant alteration in MAR. The ORX-induced increase in ES/BS was greater in the PTM and DTM than in the LVB and greater in the DTM than in the PTM.

Etidronate treatment in ORX rats significantly decreased ES/BS to a level not significantly different from that in the AMC group at all skeletal sites, resulting in complete prevention of ORX-induced cancellous BV/TV loss. In the LVB and PTM, etidronate treatment markedly decreased MAR and MS/BS and subsequently BFR/BS and BFR/BV. In the DTM, however, although MS/BS was significantly decreased by etidronate treatment, MAR was maintained.

Discussion

ORX has been reported to induce high turnover cancellous osteopenia in adult rats. In particular,
Table 2  Bone Histomorphometry of PTM

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AMC</th>
<th>ORX</th>
<th>ORX + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES/BS (%)</td>
<td>3.55 ± 1.18</td>
<td>7.02 ± 2.40</td>
<td>3.28 ± 1.71</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>5.54 ± 3.00</td>
<td>15.83 ± 5.10</td>
<td>4.84 ± 1.87</td>
</tr>
<tr>
<td>MAR (µm/d)</td>
<td>0.75 ± 0.08</td>
<td>0.76 ± 0.09</td>
<td>0.00 ± 0.09</td>
</tr>
<tr>
<td>BFR/BS (µm³/µm²/d)</td>
<td>0.043 ± 0.026</td>
<td>0.121 ± 0.040</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>BFR/BV (%/yr)</td>
<td>37.5 ± 24.5</td>
<td>102.9 ± 34.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data are means ± SD. a P < 0.001, b P < 0.01 vs AMC group, c P < 0.001 vs ORX group.

Table 3  Bone Histomorphometry of DTM

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AMC</th>
<th>ORX</th>
<th>ORX + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES/BS (%)</td>
<td>2.61 ± 0.92</td>
<td>7.09 ± 2.23</td>
<td>1.33 ± 0.56</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>4.94 ± 2.33</td>
<td>7.28 ± 3.15</td>
<td>2.47 ± 0.96</td>
</tr>
<tr>
<td>MAR (µm/d)</td>
<td>0.79 ± 0.11</td>
<td>0.79 ± 0.08</td>
<td>0.79 ± 0.10</td>
</tr>
<tr>
<td>BFR/BS (µm³/µm²/d)</td>
<td>0.040 ± 0.025</td>
<td>0.057 ± 0.026</td>
<td>0.020 ± 0.010</td>
</tr>
<tr>
<td>BFR/BV (%/yr)</td>
<td>8.47 ± 4.12</td>
<td>18.1 ± 13.5</td>
<td>7.61 ± 5.45</td>
</tr>
</tbody>
</table>

Data are means ± SD. a P < 0.001, b P < 0.05 vs AMC group, c P < 0.001, d P < 0.01 vs ORX group.

Table 4  Bone Histomorphometry of LVB

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AMC</th>
<th>ORX</th>
<th>ORX + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES/BS (%)</td>
<td>2.60 ± 0.89</td>
<td>3.86 ± 0.80</td>
<td>2.02 ± 1.01</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>5.08 ± 1.38</td>
<td>14.66 ± 4.58</td>
<td>1.25 ± 1.15</td>
</tr>
<tr>
<td>MAR (µm/d)</td>
<td>0.70 ± 0.17</td>
<td>0.73 ± 0.07</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>BFR/BS (µm³/µm²/d)</td>
<td>0.037 ± 0.019</td>
<td>0.108 ± 0.036</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>BFR/BV (%/yr)</td>
<td>25.5 ± 13.1</td>
<td>80.8 ± 25.2</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data are means ± SD. a P < 0.001, b P < 0.01, c P < 0.05 vs AMC group, d P < 0.001, e P < 0.01 vs ORX group.

Erben, et al.6 clearly demonstrated that ORX-induced osteopenia in the PTM and LVB of adult rats was associated with increased osteoclast number, osteoblast surface, bone formation rate, and activation frequency. The effect of ORX on cancellous bone in the DTM has rarely been reported. In the present study, ORX induced cancellous BV/TV loss similarly in the PTM, DTM, and LVB, which was associated with increased eroded surface and bone formation rate with increased bone turnover (ES/BS and BFR) with increased osteoblastic recruitment (MS/BS) and no significant alteration in osteoblastic activity (MAR).

Bone turnover was lower in the DTM than in the PTM and LVB and lower in the LVB than in the PTM according to the results of bone histomorphometric analysis in the AMC group. The ORX-induced increase in ES/BS was greater in the PTM and DTM than in the LVB and greater in the DTM than in the PTM. We surmise that ORX-induced body weight loss in terms of decreased body weight loading and/or the amount of progenitor cells inside the cortex participating in forming bone in the marrow cavity may somewhat influence the differential response of cancellous bone to ORX among the PTM, DTM, and LVB.

The tibia differs from the lumbar vertebra in the rat not only in the ratio of cortical to cancellous bone, but also in its position in the body where there is more body weight loading on the tibia relative to the lumbar vertebra. Furthermore, the DTM is a bony region closest to the point of weight loading (the most distal aspect of the tibia), and contains a thicker metaphyseal cortex than the PTM. The DTM, the distal aspect of the tibia, and contains a thicker metaphyseal cortex than the PTM. The tibia differs from the lumbar vertebra in the rat not only in the ratio of cortical to cancellous bone, but also in its position in the body where there is more body weight loading on the tibia relative to the lumbar vertebra.
DTM receives more body weight loading under usual activity, and furthermore contains much more progenitor cells than the PTM. Body weight change affects mechanical stress loaded on the tibia much more than that loaded on the lumbar vertebra under usual activity in rats, and also affects mechanical stress loaded on the DTM more than that loaded on the PTM. The larger number of progenitor cells in the DTM can bring about greater bone formation than in the PTM.

In fact, the effect of mechanical stress on bone is suggested to be site-specific. The effect of treadmill exercise on cancellous bone mass in rats is greater in the tibia than in the lumbar vertebrae and greater in the DTM than in the PTM. Conversely, bone loss in the weight-bearing limbs due to disuse or bed rest is greatest in bony regions closest to the point of loading (i.e., the most distal aspects). The anabolic response of cancellous bone to exercise is largest at the most distal aspects of weight-bearing limbs, and the catabolic response of bone to disuse is similarly most severe. Disuse tends to suppress bone formation and increase bone resorption. In the present study, despite lower turnover, cancellous BV/TV loss was greater in the DTM than in the PTM, probably because ORX-induced body weight loss affected the DTM more than the PTM, resulting in more accelerated bone resorption (indicated by percent increases in ES/BS). Bone formation was not suppressed by decreased body weight loading in the DTM, probably because of the effect of a larger number of progenitor cells. On the other hand, the less marked cancellous BV/TV loss in the LVB as compared with the PTM may be attributable to lower turnover and smaller body weight change effect.

Another possible explanation for the differential response of cancellous bone to ORX between the PTM and the DTM concerns the pressurization of the medullary cavity of the tibia. Bergula, et al. demonstrated that inhibition of femoral bone apposition caused by disuse could be mitigated by femoral vein ligation in rats, which significantly increased the intramedullary pressure in the femur. Thus, changes in pressure of the medullary cavity may possibly affect bone mass. The distal aspects of the hindlimbs are the furthest points from the heart and are therefore subject to higher interstitial fluid pressure. Because androgen has an anabolic effect on muscle, ORX might possibly reduce muscle volume, resulting in a reduction of blood flow and subsequent pressure of the medullary cavity of the tibia in the present study. This change in pressure of the medullary cavity of the tibia might partly affect the DTM more than the PTM.

Bisphosphonates may inhibit osteoclast-mediated bone resorption, and loss of osteoclast function and apoptosis is probably the consequence of loss of function of one or more these important signaling proteins. In particular, etidronate can be metabolically incorporated into nonhydrolyzable analogs of ATP, and intracellular accumulation of these metabolites is likely to inhibit osteoclast function. In the present study, short-term treatment with etidronate did prevent cancellous BV/TV loss in the PTM, DTM, and LVB of ORX rats. The primary effect of etidronate treatment in ORX rats was a reduction of bone resorption. However, in the LVB and PTM, etidronate treatment markedly decreased MAR, MS/BS, and subsequently BFR/BS and BFR/BV. In the DTM, on the other hand, although MS/BS was significantly decreased by etidronate treatment, MAR was maintained. This result in the DTM may be attributable to the effect of a larger number of progenitor cells in the DTM. The effect of the amount of progenitor cells may play a role in the differential response of cancellous bone to etidronate treatment in the PTM and DTM.

Major limitations of the present study are associated with use of a single dose of etidronate and short-duration of treatment. First, although the dose of etidronate was determined based on the results of a previous study, this dose might however, be so high in our rat model that a mineralization defect (indicated by a marked reduction in mineral apposition rate) was found in the PTM and LVB, even though cancellous BV/TV loss was completely prevented. Thus, a minimum effective dose should have been identified in our rat model, and it should have been determined whether use of this dose of etidronate would not cause any mineralization defect. Second, although ORX resulted in significant cancellous BV/TV loss 4 weeks postsurgery, long-term treatment with etidronate might be more useful, if this study is applied as a preclinical study. Further study of long-term duration of treatment is needed to confirm the efficacy of etidronate for ORX-induced cancellous osteopenia.

In conclusion, the results of the present study suggest that the response of cancellous bone mass to ORX appears to differ among three skeletal sites. Etidronate treatment could completely prevent ORX-induced cancellous bone loss regardless skeletal sites by suppressing bone resorption. However, suppression of bone formation in terms of osteoblastic activity by etidronate treatment was not evident only in the DTM.

References