ORIGINAL ARTICLE

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

Jun Iwamoto, Tsuyoshi Takeda and Shoichi Ichimura[1]

Department of Sports Medicine, Keio University School of Medicine, [1]Department of Orthopaedic Surgery Kyorin University School of medicine, Tokyo, Japan

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.
(Keio J Med 53 (1): 12–17, March 2004)

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-E_2 prevents age-related bone changes, adds extra bone, and improves the micro-anatomical structure by stimulating bone formation without altering bone resorption.[9] It has also been reported that prostaglandin-E_2 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]

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 exanguination under ether anesthesia at the beginning of the experi-

ment. Rats in the AMC, ORX, and ORX + E groups were labeled by subcutaneous injection of 8 mg/kg calcein 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 5th 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 according to the method of Villanueva.[14] After dehydration with ethanol and acetone, the samples were embedded in methylmethacrylate (Wako Pure Chemical, Japan). Frontal sections of the PTM and sagittal sections of the DTM and LVB were cut at 5 μm thickness using a microtome (Supercut 2050, Reichert-Jung, Heidelberg, Germany) and prepared for histomorphometric analysis of cancellous bone.

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, $μm^2$), bone volume (BV, $μm^2$), bone surface (BS, μm), eroded surface (ES, μm), single-labeled surface (sLS, μm), double-labeled surface (dLS, μm), and interlabel width (IrLWi, μm). 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, μm/day), bone formation rate/BS (BFR/BS, $μm^3/μm^2$/day), 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-

Table 1   Changes in Body Weight

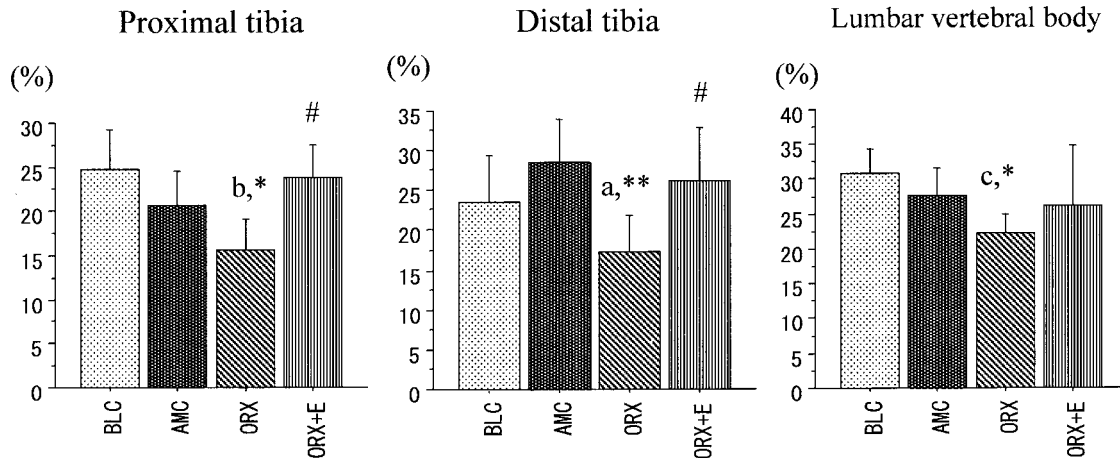|	n	Baseline	Day 7	Day 14	Day 21	Day 28	Two-way ANOVA
BLC	10	468 ± 20					
AMC	9	469 ± 21	458 ± 24	460 ± 24	468 ± 21	469 ± 24	
ORX	9	470 ± 20	434 ± 22[a]	443 ± 18	455 ± 13	456 ± 12	P < 0.05
ORX + E	7	469 ± 17	443 ± 9	445 ± 11	460 ± 13	458 ± 11	P < 0.05

[a] P < 0.05 vs AMC group.

Fig. 1   Cancellous BV/TV. Data are presented as means and SD. * P < 0.001, ** P < 0.05 vs BLC group. a: P < 0.001, b: P < 0.01, c: P < 0.05 vs AMC group. # P < 0.01 vs ORX group. BV/TV: bone volume/tissue volume.

ters 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

Parameters	AMC	ORX	ORX + E
ES/BS (%)	3.55 ± 1.18	7.02 ± 2.40[a]	3.28 ± 0.71[#]
MS/BS (%)	5.54 ± 3.00	15.83 ± 5.10[a]	0.48 ± 0.18[b,#]
MAR (μm/d)	0.75 ± 0.08	0.76 ± 0.09	0.00 ± 0.00[a,#]
BFR/BS (μm^3/μm^2/d)	0.043 ± 0.026	0.121 ± 0.040[a]	0.000 ± 0.000[b,#]
BFR/BV (%/yr)	37.5 ± 24.5	102.9 ± 34.0[a]	0.0 ± 0.0[a,#]

Data are means ± SD. [a] $P < 0.001$, [b] $P < 0.01$ vs AMC group, [#] $P < 0.001$ vs ORX group. PTM: proximal tibial metaphysis, ES: eroded surface, BS: bone surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, BV: bone volume.

Table 3 Bone Histomorphometry of DTM

Parameters	AMC	ORX	ORX + E
ES/BS (%)	2.61 ± 0.92	7.99 ± 2.23[a]	1.33 ± 0.56[#]
MS/BS (%)	4.94 ± 2.33	7.28 ± 3.15[b]	2.47 ± 0.96[b,#]
MAR (μm/d)	0.79 ± 0.11	0.79 ± 0.08	0.79 ± 0.10
BFR/BS (μm^3/μm^2/d)	0.040 ± 0.025	0.057 ± 0.026[b]	0.020 ± 0.010[b,#]
BFR/BV (%/yr)	8.47 ± 4.12	18.1 ± 13.5[b]	7.61 ± 5.45[##]

Data are means ± SD. [a] $P < 0.001$, [b] $P < 0.05$ vs AMC group, [#] $P < 0.001$, [##] $P < 0.01$ vs ORX group. DTM: distal tibial metaphysis, ES: eroded surface, BS: bone surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, BV: bone volume.

Table 4 Bone Histomorphometry of LVB

Parameters	AMC	ORX	ORX + E
ES/BS (%)	2.60 ± 0.89	3.86 ± 0.80[c]	2.02 ± 1.01[##]
MS/BS (%)	5.08 ± 1.38	14.66 ± 4.58[a]	1.25 ± 1.15[c,#]
MAR (μm/d)	0.70 ± 0.17	0.73 ± 0.07	0.00 ± 0.00[a,#]
BFR/BS (μm^3/μm^2/d)	0.037 ± 0.019	0.108 ± 0.036[a]	0.000 ± 0.000[b,#]
BFR/BV (%/yr)	25.5 ± 13.1	80.8 ± 25.2[a]	0.0 ± 0.0[c,#]

Data are means ± SD. [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$ vs AMC group, [#] $P < 0.001$, [##] $P < 0.01$ vs ORX group. LVB: lumbar vertebral body, ES: eroded surface, BS: bone surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, BV: bone volume.

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 weight-bearing limbs is in closer contact with the ground and is thus loaded more directly. Therefore, the

DTM receives more body weight loading under usual activity, and furthermore contains much more progenitor cells than the PTM.[16] 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.[17] 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).[18,19] 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.[20,21] 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.[19] Bergula, *et al.*[22] 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,[13] 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

1. Hock JM, Gera I, Fonseca J, Raisz LG: Human parathyroid hormone-(1–34) increases bone mass in ovariectomized and orchidectomized rats. Endoclinology 1998; 122: 2899–2904
2. Ke HZ, Qi H, Crawford DT, Chidsey-Frink KL, Simmons HA, Thompson DD: Lasofoxifene (CP-336, 156), a selective estrogen receptor modulator, prevents bone loss induced by aging and orchidectomy in the adult rat. Endocrinology 2000; 141: 1338–1344

3. Li M, Jee WSS, Ke HZ, Tang LY, Ma YF, Liang XG, Setterberg RB: Prostaglandin E_2 administration prevents bone loss induced by orchidectomy in rats. J Bone Miner Res 1995; 10: 66–73

4. Wakley GK, Schutte HD Jr, Hannon KS, Turner RT: Androgen treatment prevents loss of cancellous bone in the orchidectomized rat. J Bone Miner Res 1991; 6: 325–330

5. Prakasam G, Yeh JK, Chen MM, Castro-Magana M, Liang CT, Aloia JF: Effects of growth hormone and testosterone on cortical bone formation and bone density in aged orchiectomized rats. Bone 1999; 24: 491–497

6. Erben RG, Eberle J, Stahr K, Goldberg M: Androgen deficiency induces high turnover osteopenia in aged male rats: a sequential histomorphometric study. J Bone Miner Res 2000; 15: 1085–1098

7. Erben RG: Skeletal effects of androgen withdrawal. J Musculoskel Neuron Interact 2001; 1: 225–233

8. Ke HZ, Crawford DT, Qi H, Chidsey-Frink KL, Simmons HA, Li M, Jee WSS, Thompson DD: Long-term effects of aging and orchidectomy on bone and body composition in rapidly growing male rats. J Musculoskel Neuron Interact 2001; 1: 215–224

9. Ma YF, Ke HZ, Jee WSS: Prostaglandin E_2 adds bone to a cancellous bone site with a closed growth plate and low bone turnover in ovariectomized rats. Bone 1994; 15: 137–146

10. Ito H, Ke HZ, Jee WSS, Sakou T: Anabolic responses of an adult cancellous bone site to prostaglandin E_2 in the rat. Bone Miner 1993; 21: 219–236

11. Dawson AB: The age order epiphyseal union in the long bones of the albino rat. Anat Rec 1925; 31: 1–17

12. Iwamoto J, Takeda T, Katsumata T, Tanaka T, Ichimura S, Toyama Y: Effect of etidtronate on bone in orchidectomized and sciatic neurectomized adult rats. Bone 2002; 30: 360–367

13. Katsumata T, Nakamura T, Ohnishi H, Sakurama T: Intermit-tent cyclical etidronate treatment maintains the mass, structure and the mechanical property of bone in ovariectomized rats. J Bone Miner Res 1995; 10: 921–931

14. Villanueva AR: A bone stain for osteoid seams in fresh, unembedded, mineralized bone. Stain Technol 1974; 49: 1–8

15. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR: Bone histomorphometry: standardization of nomenclature, symbols, and units. J Bone Miner Res 1997; 2: 595–610

16. Ke HZ, Jee WSS, Ito H, Setterberg RB, Li M, Lin BY, Liang XG, Ma YF: Greater bone formation induction occurred in aged than young cancellous bone sites. Bone 1993; 14: 481–485

17. Iwamoto J, Yeh JK, Aloia JF: Differential effect of treadmill exercise on three cancellous bone sites in the young growing rat. Bone 1999; 24: 163–169

18. LeBlanc AD, Schneider VS, Evans HJ, Engelbretson DA, Krebs JM: Bone mineral loss and recovery after 17 weeks of bed rest. J Bone Miner Res 1990; 5: 843–850

19. Turner CH: Site-specific skeletal effects of exercise: importance of interstitial fluid pressure. Bone 1999; 24: 161–162

20. Frost MH: Structural adaptations to mechanical usage: a three-way rule for lamellar bone modeling. Comp Vet Orthop Trauma 1988; 1: 7–17

21. Zeng QQ, Jee WSS, Bigornia AE, King JG Jr, D'Souza SM, Li XJ, Ma YF, Wechter WJ: Time responses of cancellous and cortical bones to sciatic neurectomy in growing female rats. Bone 1996; 19: 13–21

22. Bergula AP, Huang W, Frangos JA: Femoral vein ligation increases bone mass in the hindlimb suspended rat. Bone 1999; 24: 171–177