Human Parathyroid Hormone-(1-38) Restores Cancellous Bone to the Immobilized, Osteopenic Proximal Tibial Metaphysis in Rats


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Running Title: PTH Restores Bone to Immobilized Rat Tibiae.

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ABSTRACT

The purpose of this study was to determine if human parathyroid hormone-(1-38) (PTH) can restore cancellous bone mass to the established osteopenic, immobilized proximal tibial metaphyses (PTM) of female rats. The right hindlimbs of six-month-old female Sprague-Dawley rats were immobilized by bandaging the right hindlimbs to the abdomen. After 30 days of right hindlimb immobilization (RHLI), the rats were subcutaneously injected with 200 μg hPTH(1-38)/kg/day for 15 (short-term) or 75 (longer-term) days. Static bone histomorphometry was performed on the primary spongiosa, while both static and dynamic histomorphometry were performed on the secondary spongiosa of the right PTM. Immobilization for 30 days without treatment decreased trabecular bone area, number and thickness in both primary and secondary spongiosa, and induced an increase in eroded perimeter and a decrease in tissue referent-bone formation rate (BFR/TV) in the secondary spongiosa. These changes reached a new steady state thereafter. Treatment with 200 μg hPTH(1-38)/kg/day for 15 days, beginning at 30 days post immobilization (IM), significantly increased trabecular bone area, thickness and number in both primary and secondary spongiosa despite continuous IM when compared to the age-related and IM controls. The short-term (15 days) PTH treatment significantly increased labeling perimeter, mineral apposition rate and BFR/TV in the secondary spongiosa and stimulated longitudinal bone growth as compared to the age-related and IM controls. PTH treatment for longer-term (75 days) further increased trabecular bone area, thickness and number as compared to aging and IM controls and short-term (15 days) PTH treated groups. The bone formation indices in the secondary spongiosa of these longer-term treated rats were lower than that of short-term (15 days) PTH treated group, but they were still higher than those of IM and age-related controls. Our findings indicate that PTH treatment stimulates cancellous bone formation, restores and adds extra cancellous bone to the established, disuse-osteopenic proximal tibial metaphysis of continuously RHLI female rats. These results suggest that PTH may be a useful agent in treating disuse-induced osteoporosis in humans.
INTRODUCTION

In a recent review Dempster and his colleagues (1), found both a number of clinical studies supporting the anabolic effect of parathyroid hormone (PTH). They reported that PTH alone or combined with 1,25(OH)\textsubscript{2}D\textsubscript{3} improved calcium and phosphate balance, increased serum 1,25(OH)\textsubscript{2}D\textsubscript{3}, stimulated bone formation and increased bone mass in postmenopausal osteoporotic patients and elderly non-osteopenic women as well as in osteoporotic men (2-11). In animal studies, PTH has been shown to stimulate bone formation and increase bone mass in intact male and female rats (12-18), prevent bone loss induced by ovariectomy (16,19-23) or orchidectomy (23), and restore bone to the osteopenic skeleton induced by ovariectomy (16,24-30), parathyroidectomy (30), hemicorpectomy (30) or lactation plus low-calcium diet (12). With these encouraging clinical and preclinical findings, we postulate that PTH should restore cancellous bone lost by immobilization. We used female rats with established IM-induced osteopenic skeleton to investigate this hypothesis.

For this study we employed the right hindlimb immobilization (RHLI) rat as an animal model of disuse osteopenia. Several studies have found that significant bone loss in the right hindlimb of RHLI rats occurred after only 1 week of immobilization, with this bone loss reaching a new steady state after 4 weeks of immobilization (31-34). In the present study, 6-month-old female rats were right hindlimb immobilized for 30 days to induce osteopenia, then treated with human parathyroid hormone (hPTH(1-38)) plus RHLI for 15 and 75 days. These two time points allowed us to assess the transient and steady state responses of the RHLI-induced osteopenic skeleton to daily PTH administration.

MATERIALS AND METHODS

Sixty-three 6-month-old virgin female Sprague-Dawley rats weighing approximately 257 g (Simonsen Laboratory, Inc., Gilroy, CA) were acclimated to local vivarium conditions (24°C and 12h/12h light-dark cycle) for 7 days. During the experimental period, the rats were allowed free
access to water and pelleted commercial natural product diet (Teklad Rodent Chow #8604, Harlan Teklad, Madison, WI), which contains 1.46% calcium, 0.99% phosphorus, and 4.96 IU/g Vit D₃. Rats were divided into four main groups and then subdivided into nine study groups (6 to 7 rats per subgroup). These groups were: (I) Age-related controls at days 0, 30, 45 and 105; (II) Right hindlimb immobilization (RHLI) controls at days 30, 45 and 105; (III) RHLI for the first 30 days, and then RHLI plus subcutaneous injections of 200 µg hPTH(1-38)/kg/day for another 15 days (sacrificed at experimental day 45); (IV) RHLI for the first 30 days, and then RHLI plus subcutaneous injections of 200 µg hPTH(1-38)/kg/day for another 75 days (sacrificed at experimental day 105) (Table I).

The right hindlimb was bound to the abdomen with elastic bandages. Four layers of this placed the hip joint in flexion and the knee and ankle joints in extension. The bandage was checked daily and replaced twice weekly (31-34). In this model, the right hindlimb is underloaded during ambulation.

We prepared our treatment solution from powdered hPTH(1-38) supplied by Bachem Co., (Torrance, CA). We dissolved in vehicle (100 ml: 192.1 mg citric acid, 331.2 mg Na₂HPO₄, 764.0 mg NaCl), and then adjusted the volume to desired concentrations. We prepared each dose daily by freezing the appropriate volume at -20°C. The rats in the PTH treated groups were subcutaneously injected with 200 µg hPTH(1-38) daily (0.5 ml/kg), while the rats in the age-related and immobilized control groups were injected with vehicle (0.5 ml/kg). The dose selected was based on the findings of Gasser and Jerome (16). The rats were weighed weekly and the volume of the injection solution was adjusted accordingly.

All rats were triple labeled. They received an injection of 90 mg/kg of Xylenol Orange (Sigma Chemical Co., St. Louis, MO) on day 30, and 25 mg/kg of Achromycin tetracycline hydrochloride (Lederle Laboratory, Pearl River, NY) at 14 and 13 days and 10 mg/kg of Calcein (Sigma Chemical Co., St. Louis, MO) at 4 and 3 days before sacrifice. The day 30 injection of Xylenol Orange was given to fluorochrome label the mineralized surface before PTH treatment (Fig. 2F).
The rats were sacrificed under ketamine hydrochloride and xylazine anesthesia via cardiac puncture. The gastrocnemius and soleus muscles were removed and weighed. The right tibiae were removed, dissected and cut into three equal parts. The proximal tibiae were fixed in 10% phosphate buffered formalin (24 to 48 hrs), 50 and 70% ethanol of 24 hrs each to remove formalin and stain in Villanueva osteochrome stain (35); (Polyscience Inc., Warrington, PA) for 5 days. The bones were then dehydrated in graded alcohols and several changes of acetone and embedded in methymethacrylate. Frontal sections of 220 μm were cut with an Isomet saw and ground to 20 μm. These 20 μm undecalcified sections were used for static and dynamic histomorphometric measurements as previously described (36-58).

A Digitizing Image Analysis System (DIAS) was used to determine the trabecular bone mass (the percent of trabecular bone area) and structural indices (the trabecular bone number and thickness) in both primary (1 mm to the growth plate-metaphyseal junction) and secondary (between 1 and 4 mm distal to the growth plate-metaphyseal junction) spongiosa of the proximal tibial metaphyses (36). We also measured the dynamic histomorphometric indices in the secondary spongiosa. These measurements included the trabecular eroded perimeter, osteoid perimeter, single-labeled perimeter, double-labeled perimeter, and interlabeling width. These measurements were used to derive indices of the percent of the osteoid perimeter, eroded perimeter, and labeled perimeter (double plus one-half single label), and mineral apposition rate, bone formation rate (bone area and tissue area referent), and longitudinal growth rate (37-41).

There was about 10 μm/day of longitudinal bone growth in the 6-month-old rats, and about 5 μm/day in the 9.5-month-old rats; therefore, after 75 days approximately 0.6 mm of new metaphysis was generated on the proximal tibia (42). By defining the primary spongiosa as that region from the growth plate-metaphyseal junction distal to 1 mm (36) and the secondary spongiosa within the area from 1 mm to 4 mm from the growth plate metaphyseal junction was analyzed. Histomorphometric measurements in the secondary spongiosa were performed on cancellous bone that was predominately remodeling and treated during the treatment period (36).
We were able to perform only static morphometric measurements in the primary spongiosa because there modeling predominated and woven bone that formed was diffusely labeled (36,43).

The differences among group means at each time period were evaluated using an analysis of variance (ANOVA) and the Fisher PLSD test (44). The differences between the means from the different time periods were analyzed using a two-tailed Student's t-test. A probability less than < 0.05 was considered significant.

RESULTS

Effects on Body Weight and Muscle Weight.

The age-related control rats gained body weight steadily during the experimental period. By days 30 and 45, the RHLI control rats had lost weight compared to age-related controls, but this loss was moderated by day 105. Body weight in the PTH-treated RHLI rats did not differ from that of the immobilization (IM) controls at day 45, and was significant higher than the IM controls and did not differ from the age-related controls by day 105 (Table 1).

There were no significant differences in either gastrocnemius or soleus muscle weights between basal and the age-matched controls (Table 1). However, both gastrocnemius and soleus muscle weights in the immobilized limbs decreased significantly compared to those of the age-related controls at all experimental time points. However, the muscle weights in the PTH-treated immobilized limbs did not significantly differ from vehicle-treated IM controls (Table 1).

Effects on Longitudinal Bone Growth Rate.

The longitudinal growth rate (LGR) in the proximal tibia significantly decreased with age, from 7.9±0.4 μm/day at 6 months (day 0) to 4.6±0.3 μm/day at 9.5 months of age (day 105) (Table 1). There was no difference in LGR between IM and age-related controls. After both 15 and 75 days of treatment, the LGR was significantly greater in the IM, PTH-treated proximal tibial than that of IM controls (Table 1).
**Qualitative Observation of Proximal Tibial Metaphyseal Cancellous Bone**

In the proximal tibial metaphysis (PTM) of the age-related control rats, the growth plate gradually thinned, while trabeculae gradually thickened with age (Figs. 1 d vs. a,b,c).

In the immobilized PTM, there was less cancellous bone mass, the trabeculae were thinner and fewer, especially in the primary spongiosa and in the junctional area of the primary and secondary spongiosa (Figs. 1 e, f, g vs. b, c, d). There was less double labeled surface (Figs. 2B vs. A) and the interlabeled distance was narrower in the immobilized PTM than in the age-related controls (Fig. 2 E vs. D).

In the 15-day-PTH-treated immobilized PTM, the cancellous bone mass was greater and the trabeculae in the primary and secondary spongiosa were thicker than in either IM or the age-related controls (Figs. 1 h vs. f and c). Cancellous bone mass continued to increase in the PTH-treated immobilized PTM for 75 days (Figs. 1 i vs. h; i vs g and d). Massive new bone formation was found in the PTH-treated immobilized PTM (Figs. 2 C vs. B and A). We deduced the bone formed by the PTH treatment was not involved in bone resorption because bone labeled with xylenol orange at the start of PTH treatment persisted (Figs. 2 F vs. D and E). Thus, a large portion of the new cancellous bone was deposited without prior bone resorption (Fig. 2 F).

**Effects on Static histomorphometry of Proximal Tibial Metaphyseal Primary Spongiosa**

Between 6 and 9.5 months of age, the trabecular bone area remained unchanged. However, there were other changes during this period. Trabecular number decreased -18% while the trabecular thickness increased +18% (Table 3 and Fig. 3).

Immobilization also induced significant decreases in trabecular bone area (-51%), thickness (-19%), and number (-39%) by day 30, and these parameters plateaued thereafter (Table 3 and Fig. 3).
In 15 day PTH-treated immobilized PTM, trabecular bone area (+51% and +249%), thickness (+30% and +81%) and number (+17% and +95%) increased significantly as compared to the age-related and IM controls, respectively (Table 3 and Fig. 3). Further, PTH treatment for a total of 75 days in the continuously immobilized PTM increased trabecular bone area (+115% and 435%), thickness (+58% and +114%) and number (+38% and +148%) as compared to the age-related and IM controls, respectively (Table 3 and Fig. 3).

*Effects on Static histomorphometry of Proximal Tibial Metaphyseal secondary Spongiosa*

Except for a thicker trabecula in the 9.5 month old secondary spongiosa, there were no significant bone mass or other architectural changes between 6 and 9.5 months of age.

Significant decreases, similar to those seen in the primary spongiosa, were found in the secondary spongiosa after 30 days of IM. These decreases included a decline in trabecular bone area (-30%), thickness (-9%), and number (-22%). After the 30 day period, these indices reached a new steady state (Table 3 and Fig. 3).

Significant improvement of the trabecular structure of secondary spongiosa appeared after 15 days of PTH treatment. When compared to the indices of the age-related and IM controls, respectively, trabecular bone area (+66% of the age-related controls, and +252% of IM controls), thickness (+39% of the age-related and +57% of IM controls), and number (+20% of the age-related, and +59% of IM controls) all increased (Table 3 and Fig. 3). After the longer 75 days, the treated groups showed further increases over age-related and IM controls, respectively, in bone mass (+246% and +419%) and structural parameters (+190% and +240% in trabecular thickness, +24% and +58% in trabecular number) (Table 3 and Fig. 3).
Effects on Dynamic Histomorphometry of Secondary Spongiosa

Between 6 and 9.5 months of age, controls had significant decreases in percent labeling perimeter (%L.Pm, -37%), mineral apposition rate (MAR, -61%), bone formation rate-tissue area referent (BFR/TV, -75%) and bone formation rate-bone area referent (BFR/BV, -78%), while there was no change in percent eroded perimeter (%Er.Pm) (Table 4 and Fig. 4).

Significant decreases in MAR (-53%), BFR/TV (-70%) and BFR/BV (-54%), and a significant increase in %Er.Pm (+34%) occurred in the immobilized PTM as compared to the age-related controls after 30 days of IM (Table 4 and Fig. 4). At day 45, the significant differences between IM and the age-related controls were limited to a decrease in BFR/TV (-33%) and an increase in %Er.Pm (+96%). All dynamic indices listed in Table 4 and Figure 4 reached a new steady state after 105 days of IM (Table 4 and Fig. 4).

Immobilized rats treated with PTH for 15 days significantly increased %L.Pm (+95% and +91%), %O.Pm (+247% and +235%), MAR (+59% and 84%), BFR/TV (+271% and +457%) and BFR/BV (+121% and +121%), when compared to IM and age-related controls. Percent eroded surface was higher than age-related controls while not different from IM controls (Table 4 and Fig. 4). Percent labeled perimeter (+81% and +77%), %O.Pm (+256% and +206%), MAR (+17% and +13%) and BFR/TV (+152% and +211%) increased significantly, and BFR/BV (-36% of IM controls) decreased significantly in the 75 days of PTH-treated rats as compared to the age-related and IM controls, respectively. Percent eroded perimeter in the 75 days PTH-treated rats did not differ from that of age-related and IM controls (Table 4 and Fig. 4).

DISCUSSION

The strategy for preventing and treating osteopenia has been based on two approaches: 1) inhibiting bone resorption with anti-resorptive agents and 2) stimulating bone formation with anabolic agents. The effects of anti-resorptive agents in depressing bone resorption and slightly increasing bone in women, for example estrogen (45-47), bisphosphonates (48-50), calcitonin
(51-52) and calcium (53), have proven beneficial. In animal experiments, these anti-resorptive agents also prevented ovariectomy- or IM-induced osteopenia (54-56). However, the use of anti-resorptive agents in restoring the lost bone in the low turnover osteopenic skeleton has been discouraging (28). The use of bone anabolic agents to stimulate bone formation and restore the lost bone appears to be a better choice to treat established osteoporosis. In this study, we successfully restored cancellous bone to the IM-induced osteopenic proximal tibial metaphysis of rats with human parathyroid hormone-(1-38).

In this study, we examined the transient and steady state skeletal responses. Initially, immobilization induced cancellous bone loss by suppressing bone formation and stimulating bone resorption. Later, the loss in trabecular bone mass reached a new steady state after 30 days of immobilization, and bone resorption and formation parameters returned to the age-related control levels by day 105. These findings are in agreement with our previous findings (31-34) and Frost's mechanostat theory (57-60).

The detailed mechanism of IM-induced bone loss remains unknown. Thompson and Rodan (61) found that indomethacin, an inhibitor of prostaglandin synthesis, prevented the IM-induced bone loss, which suggests that the increase in endogenous prostaglandin plays an important role in stimulating bone resorption. More recently, it was found that IM causes a rapid decline in parathyroid hormone-related protein (PTHrP) mRNA expression (62). This suggests that PTHrP plays a role in the decline of bone formation induced by IM. Taken together, these findings suggest that the mechanism for IM-induced bone loss must be due to multiple factors and requires much more study.

Also in the present study, we compared the different responses of proximal tibial metaphyseal primary and secondary spongiosa to IM in female rats. After 30 days of IM, the primary spongiosa lost 51% of its trabecular bone mass, while the secondary spongiosa lost less, only 31%. It was surprising to find that the positive bone balance response was the same at the primary and secondary spongiosa in PTH treated RHLI rats (Table 3). Their final trabecular bone mass in both areas was about 60%. One would expect that the more active primary spongiosa
would have been more responsive. These findings led us to postulate that the ability of the primary spongiosa to respond was dampened by its high bone turnover rate (i.e. bone modeling and remodeling rates) making less osteoblast progenitors available than those in the secondary spongiosa to react to PTH treatment.

We demonstrated that PTH not only can completely restore but over-replace cancellous bone mass to the osteopenic, continuously immobilized proximal tibial metaphysis. After 15 days of PTH treatment, all bone formation parameters increased dramatically. The increases in labeled perimeter, mineral apposition rate and bone formation rates suggest that both the recruitment and activity of osteoblasts were enhanced. Since the eroded perimeter, a bone surface resorption index, was not altered by PTH treatment, the overall increases resulted in a positive bone balance. Although the bone formation parameters fell from their 15 day treatment level after 75 days of treatment, they were still higher than those of age-related and IM controls. The 15 day level was 4 times that of the controls and the 75 day level was still 2.5 times greater than that of the controls. The decline of bone formation parameters after 75 days of PTH treatment remains unexplained.

The effect of intermittent PTH injections on bone resorption is harder to define. Previous measurements of osteoclast number and surface have reported either increases (21), remain unchanged (25,27), or decreases (20). Wronski et al. (28) compared the responses of ovariectomy (OVX) with OVX rats in early stages of PTH treatment. They found that in the OVX controls, the bone formation rate increased. There was no difference in the osteoclast surface at 35 days, but by 105 days, the osteoclast surface increased. However, during the same period, bone formation rate declined. In contrast, our study showed that the 15 day and 75 days of PTH treatments did not alter the eroded surface when compared to those of IM controls. The earlier depression of resorption and continued stimulated bone formation created a positive bone balance and marked augmentation of bone mass in PTH-treated immobilized proximal tibial metaphysis (PTM).

This study found that PTH treatment stimulated longitudinal bone growth in the immobilized PTM. This same finding has also been reported for PTH-treated ovariectomized rats (28). PTH-stimulated longitudinal bone growth may have contributed, at least in part, to a greater
cancellous bone mass especially in the primary spongiosa. This hypothesis is supported by the findings in the chick growth plate culture study of Crabb et al., where PTH dramatically increased thymidine incorporation and the synthesis of proteoglycan (63). These results suggest a possible regulatory role for PTH in endochondral ossification.

In conclusion, our findings indicate that PTH is a powerful anabolic bone agent that can overcome disuse-induced bone loss. These results further suggest that PTH may be an effective treatment for disuse-induced osteopenia in humans.
ACKNOWLEDGMENT

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REFERENCES


18. Oxlund H, Ejersted C, Andreassen TT, Torring O, Nilsson MHL Parathyroid hormone (1-34) and (1-38) stimulate cortical bone formation both from periosteum and endosteum. Calcif Tissue Int 53:394-399.


52. Hodsman AB, Fraher LJ 1990 Biochemical responses to sequential human parathyroid hormone (1-38) and calcitonin in osteporotic patients. Bone Min 9: 137-152.


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FIGURE LEGENDS

Fig. 1 Microphotographs of proximal tibial metaphyses from the age-related controls at 6 (a), 7 (b), 7.5 (c) and 9.5 (d) months of age, immobilization controls (IM) at days 30 (e), 45 (f) and 105 (g), and 200 μg PTH/kg/day treated immobilization proximal tibial metaphysis (IM+PTH) at 15 (h) and 75 (i) days post-treatment. See text for explanation. X 4.

Fig. 2 Microphotographs of proximal tibial metaphyses (PTM) and representative trabeculae from the secondary spongiosa of the age-related controls (A and D), immobilization controls (B and E), and 200 μg PTH/kg/day treated immobilized proximal tibial metaphysis for 75 days (C and F). Cancellous bone mass was less and trabeculae were thinner and fewer in the immobilization control (B) than in age-related control (A). Greater cancellous bone mass and thicker, denser trabeculae were observed in the primary and secondary spongiosa of 75-day-PTH-treated immobilized PTM (C, F) than in immobilized (B and E) and the age-related controls (A and D). There was more double labeled trabecular surface and a wider interlabel distance in the 75-day-PTH-treated immobilized PTM (C, F) than in immobilized (B and E) and the age-related controls (A and D). Xylenol orange (XO), which was given to all rats at the onset of PTH or vehicle administration, and tetracycline and calcein (DL) were found in PTH-treated immobilized PTM (F), while only tetracycline and calcein (DL) can be found in the age-related (D) and immobilized (E) controls. Magnifications: A - C, X12; D - F, X100.

Fig. 3 Trabecular bone area changes in primary (Zone I, A) and secondary (Zone II, B) spongiosa of the age-related controls (aging), the immobilization controls (IM) and the PTH-treated immobilized proximal tibial metaphysis (IM+PTH). *: p < 0.05 vs. basal; #: p < 0.05 vs. aging; @: p < 0.05 vs. IM.

Fig. 4 Eroded perimeter (A), labeling perimeters (B), mineral apposition rate (C) and bone formation rate-tissue referent (D) changes in secondary spongiosa of the age-related controls (aging), the immobilization controls (IM) and the PTH-treated immobilized
proximal tibial metaphysis (IM+PTH). *: p < 0.05 vs. basal; #: p < 0.05 vs. aging; @: p < 0.05 vs. IM.
<table>
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<th>105</th>
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<td>7</td>
<td>7.5</td>
<td>10.5</td>
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<tr>
<td>Aging Controls</td>
<td>7†</td>
<td>..............</td>
<td>7</td>
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<td>IM Controls</td>
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<tr>
<td>IM+PTH</td>
<td>..........................</td>
<td>7</td>
<td>..........................</td>
<td>7</td>
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†: number of rats will be autopsied; ........: no treatment; ・・・・: vehicle treatment; ・・・・: right hindlimb immobilization (IM) plus no treatment; ♥♥: right hindlimb immobilization (IM) plus vehicle treatment; ・・・・: right hindlimb immobilization (IM) plus 200 μg/kg/day of hPTH-(1-38) treatment.
Table 2. Changes in Body Weight, Muscle Weight and Longitudinal Growth Rate

<table>
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<tr>
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<th>Body Weight n (g)</th>
<th>Muscle Weight</th>
<th>Longitudinal Growth Rate (µm/day)</th>
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<td></td>
<td>Gastrocnemius (g)</td>
<td>Soleus (g)</td>
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<td>0.67 ± 0.04 *,#</td>
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<td>1.81 ± 0.05</td>
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<td>0.82 ± 0.09 *,#</td>
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<tr>
<td>IM+PTH †</td>
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<td>0.69 ± 0.02 *,#</td>
<td>0.04 ± 0.01 *,#</td>
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<td><strong>Day 105</strong></td>
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<td>IM+PTH ††</td>
<td>6 279 ± 6.9 @</td>
<td>1.26 ± 0.06 *,#</td>
<td>0.06 ± 0.01 *,#</td>
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Mean±SEM; n: number of rats in each group; IM: immobilization.

†: immobilized for the first 30 days, and then immobilization plus 200 µg hPTH-(1-38)/kg/day for 15 days.

††: immobilized for the first 30 days, and then immobilization plus 200 µg hPTH-(1-38)/kg/day for 75 days.

*: p < 0.05 vs basal controls; #: p < 0.05 vs aging controls; @: p < 0.05 vs IM controls.
Table 3. Changes in Bone Mass and Structural Indices of Primary and Secondary Spongiosa of Proximal Tibial Metaphyses

<table>
<thead>
<tr>
<th>Groups</th>
<th>Primary Spongiosa (Zone I)</th>
<th>Secondary Spongiosa (Zone II)</th>
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<tr>
<td></td>
<td>Trabecular Bone Area (%)</td>
<td>Trabecular Thickness (µm)</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Controls</td>
<td>27.4 ± 1.2</td>
<td>60.7 ± 1.3</td>
</tr>
<tr>
<td>Day 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging Controls</td>
<td>26.1 ± 1.1</td>
<td>62.7 ± 2.4</td>
</tr>
<tr>
<td>IM Controls</td>
<td>12.9 ± 1.2 *,#</td>
<td>50.3 ± 1.9 *,#</td>
</tr>
<tr>
<td>Day 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging Controls</td>
<td>24.0 ± 2.0</td>
<td>68.8 ± 2.3</td>
</tr>
<tr>
<td>IM Controls</td>
<td>10.4 ± 2.0 *,#</td>
<td>49.5 ± 2.7 *,#</td>
</tr>
<tr>
<td>IM+PTH †</td>
<td>36.3 ± 1.3 *,#,@</td>
<td>89.4 ± 2.3 *,#,@</td>
</tr>
<tr>
<td>Day 105</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging Controls</td>
<td>26.8 ± 2.0</td>
<td>71.9 ± 2.3</td>
</tr>
<tr>
<td>IM Controls</td>
<td>10.8 ± 1.2 *,#</td>
<td>52.9 ± 2.4 *,#</td>
</tr>
<tr>
<td>IM+PTH ††</td>
<td>57.7 ± 2.6 *,#,@</td>
<td>113.4 ± 4.7 *,#,@</td>
</tr>
</tbody>
</table>

Mean±SEM; n: number of rats in each group; IM: immobilization.

†: immobilized for the first 30 days, and then immobilization plus 200 µg hPTH-(1-38)/kg/day for 15 days.

††: immobilized for the first 30 days, and then immobilization plus 200 µg hPTH-(1-38)/kg/day for 75 days.

*: p < 0.05 vs basal controls; #: p < 0.05 vs aging controls; @: p < 0.05 vs IM controls.
Table 4. Changes in Dynamic Histomorphometry of Secondary Spongiosa of Proximal Tibial Metaphyses

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eroded Perimeter (%)</th>
<th>Labeling Perimeter (%)</th>
<th>Osteoid Perimeter (%)</th>
<th>Mineral Apposition Rate (μm/d)</th>
<th>Bone Formation Rate/TV (%/yr)</th>
<th>Bone Formation Rate/BV (%/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Controls</td>
<td>0.9±0.1</td>
<td>24.6±1.9</td>
<td>9.7±1.7</td>
<td>1.8±0.1</td>
<td>79.4±4.8</td>
<td>509±42</td>
</tr>
<tr>
<td>Aging Controls</td>
<td>1.0±0.3</td>
<td>16.3±2.3</td>
<td>10.3±1.8</td>
<td>1.3±0.1</td>
<td>45.8±10.5</td>
<td>260±50</td>
</tr>
<tr>
<td>IM</td>
<td>1.4±0.2 *,#</td>
<td>15.0±1.4 *</td>
<td>15.5±2.7 *</td>
<td>0.6±0.0 *,#</td>
<td>13.9±0.9 *,#</td>
<td>119±13 *,#</td>
</tr>
<tr>
<td><strong>Day 45</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging Controls</td>
<td>0.8±0.1</td>
<td>17.6±0.6 *</td>
<td>11.7±2.7</td>
<td>0.7±0.0 *</td>
<td>22.6±1.8 *</td>
<td>150±13 *</td>
</tr>
<tr>
<td>IM controls</td>
<td>1.8±0.3 *,#</td>
<td>17.9±0.7 *</td>
<td>12.1±1.4</td>
<td>0.6±0.0 *</td>
<td>15.0±1.8 *,#</td>
<td>150±13 *</td>
</tr>
<tr>
<td>IM+PTH †</td>
<td>1.1±0.2 #</td>
<td>34.3±2.0 *,#,@</td>
<td>40.6±2.4 *,#,@</td>
<td>1.2±0.0 *,#,@</td>
<td>83.8±4.9 #,#,@</td>
<td>331±14 *,#,@</td>
</tr>
<tr>
<td><strong>Day 105</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aging Controls</td>
<td>0.8±0.08</td>
<td>15.4±1.3 *</td>
<td>6.3±0.6 *</td>
<td>0.7±0.0 *</td>
<td>19.8±2.2 *</td>
<td>111±11 *</td>
</tr>
<tr>
<td>IM controls</td>
<td>1.0±0.13</td>
<td>15.7±1.8 *</td>
<td>7.3±1.0</td>
<td>0.7±0.0 *</td>
<td>16.1±1.8 *</td>
<td>135±12 *</td>
</tr>
<tr>
<td>IM+PTH ††</td>
<td>0.9±0.12</td>
<td>27.8±4.3 #,@</td>
<td>22.3±6.3 #,@</td>
<td>0.8±0.0 *,#,@</td>
<td>50.0±6.0 *,#,@</td>
<td>86.9±14 *,#,@</td>
</tr>
</tbody>
</table>

Mean±SEM; n: number of rats in each group; IM: immobilization.

†: immobilized for the first 30 days, and then immobilization plus 200 μg hPTH-(1-38)/kg/day for another 15 days;
††: immobilized for the first 30 days, and then immobilization plus 200 μg hPTH-(1-38)/kg/day for another 75 days;
*: p < 0.05 vs basal controls; #: p < 0.05 vs aging controls; @: p < 0.05 vs IM controls.
Fig. 1  Microphotographs of proximal tibial metaphyses from the age-related controls at 6 (a), 7 (b), 7.5 (c) and 9.5 (d) months of age, immobilization controls (IM) at days 30 (e), 45 (f) and 105 (g), and 200 µg PTH/kg/day treated immobilization proximal tibial metaphysis (IM+PTH) at 15 (h) and 75 (i) days post-treatment. See text for explanation. X 4.
Microphotographs of proximal tibial metaphyses (PTM) and representative trabeculae from the secondary spongiosa of the age-related controls (A and D), immobilization controls (B and E), and 200 μg PTH/kg/day treated immobilized proximal tibial metaphysis for 75 days (C and F). Cancellous bone mass was less and trabeculae were thinner and fewer in the immobilization control (B) than in age-related control (A). Greater cancellous bone mass and thicker, denser trabeculae were observed in the primary and secondary spongiosa of 75-day-PTH-treated immobilized PTM (C, F) than in immobilized (B and E) and the age-related controls (A and D). There was more double labeled trabecular surface and a wider interlabel distance in the 75-day-PTH-treated immobilized PTM (C, F) than in immobilized (B and E) and the age-related controls (A and D). Xylenol orange (XO), which was given to all rats at the onset of PTH or vehicle administration, and tetracycline and calcein (DL) were found in PTH-treated immobilized PTM (F), while only tetracycline and calcein (DL) can be found in the age-related (D) and immobilized (E) controls. Magnifications: A - C, X12; D - F, X100.
Fig. 3. Trabecular bone area changes in primary (Zone I, A) and secondary (Zone II, B) spongiosa of the age-related controls (aging), the immobilization controls (IM) and the PTH-treated immobilized proximal tibial metaphysis (IM+PTH). *: p < 0.05 vs. basal; #: p < 0.05 vs. aging; @: p < 0.05 vs. IM.
Fig. 4 Eroded perimeter (A), labeling perimeters (B), mineral apposition rate (C) and bone formation rate-tissue referent (D) changes in secondary spongiosa of the age-related controls (aging), the immobilization controls (IM) and the PTH-treated immobilized proximal tibial metaphysis (IM+PTH). *: p < 0.05 vs. basal; #: p < 0.05 vs. aging; @: p < 0.05 vs. IM.