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Role of Parathyroid Hormone in the Mechanosensitivity of Fracture Healing

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Abstract

The mechanical environment at a fracture site can influence the course of healing. Intermittent parathyroid hormone (PTH) has been shown to accelerate fracture healing. Intact bone models show that mechanical loading and PTH have a synergistic beneficial effect on osteogenesis. We hypothesized that PTH and mechanical loading would have a similar synergistic effect on fracture healing. Eighty mice underwent surgical osteotomy and intramedullary nailing of the tibia. The mice were divided into four groups: one underwent daily loading, one received daily subcutaneous PTH injections (30 µg/kg/day), one received both loading and PTH, and a control group received sham loading and vehicle injection. Daily loading was applied to the ends of the tibia with an external loading device for 2 weeks. Fracture healing was assessed by microcomputed tomography, histology, and biomechanical testing. The group with both loading and PTH had increased osteoblast and osteoclast activity and was the only group with a significantly larger callus mineral density and bone volume fraction. The PTH only group had significantly more osteoid in the callus compared to the control group, indicating enhanced early osteoblast activity. This group also had a significantly higher bone mineral content and total bone volume compared to controls. The group that received loading as the only intervention had significantly greater osteoclast activity versus controls. The contribution of loading and PTH administration to the fracture healing cascade indicates a synergistic effect. This finding may be of potential clinical utility when weight bearing is utilized to stimulate lower extremity fracture healing.

Keywords

parathyroid hormone; fracture healing; mouse tibia; in vivo mechanical loading; microcomputed tomography; mechanosensitivity

INTRODUCTION

Fracture healing involves a complex interplay of many local and systemic factors. The mechanical environment of a healing fracture is well known to influence fracture healing.^{1–6} Specifically, cyclic compression of fractures can enhance fracture healing, leading to earlier and more advanced callus formation under appropriate fixation rigidity and fracture strain conditions.^{1,4,7–9} For example, studies in sheep have demonstrated that dynamization of external fixation leads to accelerated fracture healing compared to rigid fixation.^{4,10} We

recently applied a noninvasive cyclic loading protocol to mouse tibial fractures and found increased bone strength following loading at low loads initiated several days post-fracture.¹

Several cellular and bone adaptation studies have found that parathyroid hormone (1–34; PTH) has an additive effect with mechanical loading on osteoblastic activity and bone formation.^{11–19} In intact bone, the combination of loading and PTH stimulates greater bone formation than either individual treatment, possibly because these two interventions work through converging biochemical pathways.^{11–13,15} Furthermore, in parathyroidectomized animals, physiologic levels of PTH are required for mechanical sensitivity of osteoblasts.¹¹ PTH has a beneficial effect on fracture healing as well, with several recent reports describing accelerated fracture repair with intermittent PTH treatment.^{20–26} Therefore, we hypothesized that simultaneous PTH administration and loading have a synergistic effect on fracture healing.

METHODS

Eighty C57Bl/J6 male mice, 10 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME) and acclimated in our facilities. All mice underwent surgical tibial osteotomy and intramedullary nailing following a previously described protocol¹ approved by our Institutional Animal Care and Use Committee. Briefly, an incision was made over the dorsal aspect of the knee, and a 25-gauge needle was used to bore a hole in the proximal tibia. An oscillating saw was used to create a transverse osteotomy 5 mm distal to the patellar tendon insertion. A 27-gauge needle was then inserted across the osteotomy into the distal fragment and cut flush at the insertion hole. Postoperative radiographs were used to verify that the pins were properly positioned, and that all fibulas remained intact to ensure similar rotational stability. Buprenorphine was administered ad libitum for pain relief, and no anti-inflammatory medications were used. All mice were allowed unrestricted cage activity, and the animals did not appear to favor the non-operated limb.

Following surgery, the mice were randomly divided into four groups ($n = 20$ per group): one group underwent daily loading; one received daily subcutaneous PTH injections; one received both loading and PTH; and a fourth group served as a control. The loading-only group also received injection with a vehicle, and the PTH-only group also underwent sham loading. The control group received sham loading and vehicle injection.

Cyclic mechanical loads were applied daily (5×/week) to the fractured tibia with a noninvasive external loading device.^{1,27} Loading began after a 4-day delay postfracture and continued for 2 weeks. A total of 100 load cycles were applied per day at a rate of 1 Hz and with a peak amplitude of 0.5 N using a triangle waveform; these parameters are osteogenic in the fractured mouse tibia.¹ Aliquots of synthetic 1–34 rhPTH (Bachem, Torrance, CA) were prepared with a vehicle of 1M acetic acid and 1% heat inactivated mouse serum to achieve a PTH concentration of 30 µg/kg per dose. This is a moderate dose relative to previous animal fracture healing studies using PTH, and has been shown to accelerate fracture healing in rats.^{24,25} A single dose of the PTH solution or an equal volume of acetic acid/serum vehicle (depending on the experimental group) was given subcutaneously in the scapular region just prior to actual or sham loading.

After completion of the 2-week intervention period (postfracture day 18), the animals were killed by carbon dioxide inhalation. Immediately following euthanasia, the tibiae were dissected free of soft tissue, and the intramedullary pins were removed. The specimens were frozen in moist gauze at -20°C in preparation for analysis.

Quantitative microcomputed tomography (microCT; MS-8 small animal scanner, GE Healthcare, formerly EVS Corporation, Ontario, Canada) was performed on all specimens to determine the extent of mineralized tissue formation. Tibiae were placed in a saline-filled scan

tube and scanned at 80 V and 80 μ A. Each scan included a phantom containing air, saline, and an SB-3 bone analogue (1.18 g/cc) for calibration of image Hounsfield Units to tissue mineral density. Individual CT slices were reconstructed using a modified Parker algorithm,^{28,29} with a resolution of 24 μ m. Images were thresholded using 25% of the mineral attenuation of the cortical bone for each specimen. Regional analyses of the thresholded scans were performed using the system software (Micro-View, GE Healthcare Technologies, Waukesha, WI), and a three-dimensional volume of interest was constructed around the callus using slice-wise registration of the callus boundaries. Bone mineral content, bone mineral density, bone volume fraction, and callus volume were determined. Then, to estimate union, mid-sagittal and mid-coronal images of each callus were blindly evaluated and the number of bridged cortices was determined.³⁰

Five specimens from each group underwent histological tissue analysis. Immediately following harvesting and cleaning of muscle tissue, the bones were preserved in 10% phosphate-buffered formalin for 2 days at 4°C. Samples were then washed overnight, dehydrated with alcohols followed by xylene, and embedded in methyl methacrylate.³¹ Calcified tissues were then sectioned to 5- to 7- μ m thick slices with a tungsten carbide blade on a microtome (Reichert-Jung Ultracut E). Sections were subsequently stained with von Kossa to evaluate mineralized tissue in the callus, with Goldner's Trichrome to quantify the presence of osteoid, and with tartrate-resistant acid phosphatase (TRAP) antibody to assess osteoclast activity.³² The amount of mineralized tissue and osteoid as a percentage of the entire callus, as well as the number of osteoclasts per high power field (0.046 mm²) were quantified by a blinded investigator using a morphometric analysis system (Bioquant, Nashville, TN).

The remaining specimens in each group were tested to failure in four-point bending on a precision electromagnetic-based load frame to evaluate callus mechanical strength (EnduraTEC ELF 3200, Bose Corporation, Minnetonka, MN). Tibiae were placed with the flat anteromedial surface down on the lower supports. The lower supports were set 8.4 mm apart, and the upper load points were set 3.5 mm apart, centered over the lower supports and spanning the callus. Displacement was applied at 0.033 mm/s until failure occurred. The bending moment to failure (N/mm) and stiffness (N/mm²) were calculated from the resulting load-deflection curves.

The mean and standard deviation were calculated for each measurement for each group. One-way ANOVA tests, followed by Bonferroni post hoc tests, were used to compare the means of each group, using p -value < 0.05 to denote significance (PASS 6.0, NCSS, Kaysville, UT). The number of mice per experimental group was determined from a power analysis conducted prior to the experiment. With 15 mice per group, there was over 80% power to detect a difference in mechanical properties among groups of 1.2 times the pooled standard deviation using a one-factor analysis of variance design (α = 0.05) based on previous studies in our laboratory. An additional five animals were added per group to quantify histological comparisons.

RESULTS

The percentage of osteoid on the trabecular surfaces of both the PTH only (45.6% $\pm$ 8.4%) and the loading + PTH groups (47.1% $\pm$ 7.1%) was significantly greater than that observed for the control group (30.6 $\pm$ 4.0%, p < 0.05 for both), indicating increased early bone healing (Fig. 1). Both the loading only and the loading + PTH groups had greater numbers of active osteoclasts than the controls: 11.1 $\pm$ 1.1 and 11.2 $\pm$ 1.9, respectively, versus 8.1 $\pm$ 0.9 for the controls (p < 0.05 for both, Fig. 2). The percentage of callus mineralization ranged from 32.8% $\pm$ 9.0% for the control group to 40.1% $\pm$ 5.3% for the loading + PTH group, but no significant differences were detected among the four experimental groups. Similarly, no differences were

found in the percentage of cartilage in the calluses among the four groups, and, as expected, very little of the callus (from 2%–4%) was composed of cartilage at 18 days postfracture.^{33, 34}

On microCT analysis, the loading + PTH group had a significantly greater bone mineral density than the controls ($p < 0.05$), and greater bone volume fraction than both the control group ($p < 0.02$) and the loading-only group ($p < 0.05$). The PTH-only group had a significantly greater bone mineral content and bone volume compared to the controls ($p < 0.05$, Table 1, Fig. 3), but the bone mineral density and bone volume fraction were not different. No statistical differences in callus volume were seen among the four groups (Fig. 4, Table 1). The average number of cortices that were clearly bridged on microCT imaging was 1.8 for the control group, 2.0 for the loading-only group, 1.8 for the PTH-only group, and 1.9 for the loading + PTH group (range from 0–3 for all four groups, $p > 0.5$).

Analysis of the biomechanical properties of the specimens revealed no significant differences in maximum bending moment to failure or bending stiffness among the groups (Table 1).

DISCUSSION

In vitro cellular studies have offered mechanistic evidence that mechanical stimulation and PTH work synergistically to increase osteoblast function and bone formation, but this effect has not previously been demonstrated in an in vivo fracture model. Our data indicate that cellular activity during fracture healing was stimulated more with a combination of loading and PTH than with either individual treatment alone, suggesting a synergistic effect. The loading + PTH group was the only group with significantly increased osteoblast and osteoclast activity, as well as a greater bone mineral density and bone volume fraction relative to controls.

The apparent accelerated fracture healing that we found with this treatment combination is supported by previous studies using non-fracture models. In a rat vertebrae adaptation study, loading or PTH alone each significantly increased bone formation and mineral apposition rate, but a further increase occurred when the treatments were combined.¹³ The authors concluded that PTH may augment osteoblastic bone formation at regions of high strain by sensitizing cells to mechanical forces.¹³ Following osteopenia induced in rat tibiae, remobilization or PTH alone restores bone mass to a certain degree, but combined mechanical stimulation and PTH produces greater bone formation.¹⁵

Evidence is accruing that mechanical loading and PTH may work through similar pathways, which may partially explain the synergistic effects of combining the two treatments in adaptation studies. Studies using osteoblast cell lines have reported that strain application and PTH activate common second-messenger systems in cells.^{16–18,35} Increases in prostaglandin E2 levels occur following both mechanical stimulation and PTH treatment, suggesting converging pathways of the activating mechanisms,¹⁶ which may ultimately affect cellular calcium channel permeability^{18,19,35} and intracellular cAMP levels.¹⁷ Cyclooxygenase-2 (COX-2), which is a mediator of bone formation, is increased by both mechanical stimulation and PTH, and the application of both produces an additive effect and greater COX-2 levels than either treatment alone.¹⁹ Pretreatment with PTH lowers the threshold of osteoblastic response to loading and synergistically increases the anabolic response.^{19,36} Of the various local mediators involved in fracture healing, insulin-like growth factor 1 (IGF-1) also has a central role early in the fracture healing cascade,³⁷ including matrix synthesis,³⁸ particularly in periosteal cells, mesenchymal cells, proliferating chondrocytes, preosteoblasts, and osteoblasts.^{23,38} Both PTH and increased mechanical loading cause increases in IGF-1 expression.^{39–41}

Our loading + PTH group had significantly greater osteoblast and osteoclast activity and was also the only group to have an increased mineralization density and bone volume fraction, implying that this group had the most advanced, mature callus. The loading-only group had greater osteoclast, but not osteoblast, activity, whereas the PTH only group had greater osteoblast, but not osteoclast, activity compared to the controls. Thus, we can deduce that mechanical loading contributed a substantial component of the increased osteoclast activity to the acceleration of fracture healing and later remodeling. Direct stimulation of osteoclast differentiation by mechanical loading has been demonstrated under controlled conditions in cell culture.⁴² In intact bone adaptation, the major cellular response is upregulation of osteoblast activity, leading to new bone formation.^{10,43–47} However, the fracture healing environment is distinct in that osteoclast presence and function is a necessary component of the normal progression of the fracture healing cascade.^{48–50}

Conversely, both PTH groups (PTH only and loading + PTH) had significantly greater osteoid present compared to the control, whereas no difference was seen between the loading-only and control groups. Additionally, the PTH-only group had a greater bone mineral content relative to the control group, a finding corroborated by previous studies.^{20,26,51} These findings imply that the early phases of fracture healing were accelerated due to PTH administration. On a cellular level, PTH suppresses osteoblast apoptosis and results in prolonged and enhanced bone formation.⁵² In a rat femoral fracture model with a similar dose of PTH as our dose, early osteoid deposition and subsequent mineralization were accelerated without affecting osteoclast density or fracture remodeling.²⁴

The ability of immature callus tissue to achieve structural integrity requires early remodeling by the coupled action of both osteoblasts and osteoclasts.^{49,53} Early studies on the fracture healing effects of bisphosphonates, which pharmacologically inhibit osteoclast function, have demonstrated impaired bone mineralization,^{54,55} and more recent studies on newer bisphosphonates have also indicated that callus development in animal fracture models may be delayed.^{53,56} Temporally, the earliest major contribution of the osteoclast cell lineage to fracture healing is during the remodeling stage, when woven bone is replaced with lamellar bone. We evaluated healing 18 days postfracture, which most closely corresponds to the transition between the soft callus phase and the hard callus phase, when woven bone tissue continues to replace cartilage and remodeling of woven bone to lamellar bone is just beginning.^{34,50} The small amount of cartilage present in the calluses at this time point probably precludes accurate analysis of the effects of these interventions on cartilage deposition.^{33,34}

Our study had limitations. We cannot explain the lack of differences in biomechanical properties among our test groups, despite histologically more advanced and radiographically denser calluses. A previous study found mechanically stronger fracture calluses with PTH administration 21 days postfracture,²⁴ although when the strength was normalized to callus size, no mechanical difference existed in the osseous tissue.^{26,51} The strength increase may depend upon accrual of sufficient callus volume, which may not have occurred by 18 days postfracture. Alternatively, the increased mineral content may not have organized adequately to have a beneficial mechanical effect; this effect may not occur until between 6 to 12 weeks after fracture.^{22,25} Mechanical properties may appear to be the most clinically relevant outcome, but experimentally the variance is generally larger than with measurements of bone mineral content and density, which may more accurately reflect smaller changes in bone formation.²⁴ Another limitation in our study was the single time point studied. Our goal was to examine the effects of these treatments on the early phases of fracture healing, but nonetheless the choice precludes us from making conclusions on temporal and the long-term effects. Finally, we used histomorphometry to quantify several tissue and cell types in the calluses, but did not use a validated method to determine global fracture healing, which may have limited the applicability of our data. Despite this, we believe the significant differences

in osteoid and osteoclasts we found were important indicators of altered fracture healing courses between groups.

In summary, intermittent daily PTH administration for 18 days following fracture increased bone formation in a healing diaphyseal midshaft fracture in the mouse tibia, with increased osteoid deposition histologically and greater callus mineralization on microCT. Addition of a daily cyclic compressive loading regimen across the fracture site further accelerated fracture healing. The combination of the two interventions led to calluses with greater osteoblast and osteoclast activity and increased mineral density and bone volume fraction, all indicating more advanced fracture healing. Further analyses at later time points will clarify the structural implications of these histological and radiographic findings, but loading and PTH appear to act synergistically to accelerate bone healing.

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Figure 1.
Mean and standard deviations of the osteoid area percentage in the calluses.

Figure 2.
Mean and standard deviations of the osteoclast number and activity per area in the calluses.

Figure 3.

Mean and standard deviations of the bone mineral content and bone mineral density measured on microCT.

Figure 4.
The mean and standard deviations of the callus volumes for each group.

Table 1

Groupwise Comparisons of Bone Healing

	Group			
	Control	Load Only	PTH Only	Load + PTH
Percentage osteoid	30.6	39.1	45.7*	47.1*
SD	4.0	7.3	8.4	5.3
Percentage mineralized tissue	32.8	39.9	38.3	40.8
SD	9.0	8.6	10.9	5.3
Osteoclast number	8.1	11.1	10.4*	11.2*
SD	0.9	1.1	2.0	1.9
BMD (mg/cm ³)	470.3	506.2	493.4	546.6*
SD	66.4	59.8	68.4	51.6
BMC (mg)	1.15	1.32	1.60*	1.45
SD	0.35	0.38	0.50	0.29
Bone volume (mm ³)	18.71	20.46	26.75*	21.44
SD	8.00	5.79	8.62	5.96
Bone volume fraction	0.773	0.778	0.815	0.823*,**
SD	0.039	0.038	0.039	0.045
Callus volume (cm ³)	0.024	0.026	0.033	0.026
SD	0.011	0.007	0.011	0.007
Failure moment (N/mm)	16.5	16.1	18.3	16.5
SD	5.1	6.3	11.3	6.2
Stiffness (N/mm ²)	520.3	606.5	447.7	535.5
SD	157.0	205.8	218.3	187.4

* $p < 0.05$ vs. control group.

** $p < 0.05$ vs. load-only group.