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The Effectiveness of Human Parathyroid Hormone and Low-Intensity Pulsed Ultrasound on the Fracture Healing in Osteoporotic Bones

KARIMA Q. MANSJUR,[1] SHINGO KURODA,[1] TAKASHI IZAWA,[1] YUICHI MAEDA,[1] MINAMI SATO,[1] KEIICHIRO WATANABE,[1] SHINYA HORIUCHI,[1] and EIJI TANAKA[1,2]

[1]Department of Orthodontics and Dentofacial Orthopedics, Institute of Health Biosciences, Tokushima University Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan; and [2]Department of Orthodontics, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia

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Abstract—Osteoporotic fracture has become a major public health problem, and until today, the treatments available are not satisfactory. While there is growing evidence to support the individual treatment of parathyroid hormone (PTH) administration and low-intensity pulsed ultrasound (LIPUS) exposure as respectively systemic and local therapies during osteoporotic fracture healing, their effects have not yet been investigated when introduced concurrently. This study aimed to evaluate the effects of combined treatment with PTH (1–34) and LIPUS on fracture healing in ovariectomized (OVX) rats. Thirty-two, 12-week-old female Sprague–Dawley rats were OVX to induce osteoporosis. After 12 weeks, the rats underwent surgery to create bilateral mid-diaphyseal fractures of proximal tibiae. All animals were randomly divided into 4 groups ($n = 8$ for each): control group as placebo, PTH group, LIPUS group, and combined group. PTH group had PTH administration at a dose of 30 μg/kg/day for 3 days/week for 6 weeks. LIPUS group received ultrasound 5 days/week for 20 min/day for 6 weeks and combined group had both PTH administration and LIPUS exposure for 6 weeks. Fracture healing was observed weekly by anteroposterior radiography and micro-CT. Five weeks after the fracture, the tibia were harvested to permit histological assessments and at week 6, for mechanical property of the fracture callus. Micro-CT showed that the PTH and combined groups exhibited significantly higher BMD and trabecular bone integrity than control group at weeks 4–6. Radiography, fracture healing score and mean callus area indicated that the combined group revealed better healing processes than the individual groups. Mechanically, bending moment to failure was significantly higher in LIPUS, PTH and combined groups than in control group. These data suggest that the combined treatment of PTH and LIPUS have been shown to accelerate fracture bone healing and enhance bone properties rather than single agent therapy, and may be considered as a treatment remedy for fracture healing in postmenopausal osteoporosis.

Keywords—Low-intensity ultrasound, Parathyroid hormone, Osteoporotic fracture, Bone healing, Mechanical stimulation.

INTRODUCTION

Osteoporosis is a major public health problem which is characterized by the systemic damage of bone mass and microarchitecture, resulting in the fracture of the bones.[24] While the number of osteoporosis worldwide is increasing year by year, it is estimated that one in three women and one in five men over the age of fifty will develop osteoporotic fractures.[29] As osteoporosis may cause bone rupture even under mild trauma, maintain and treating osteoporosis to prevent fractures is of great importance. Although major osteoporotic fracture rates declined from 1996 to 2006,[16] an osteoporotic fracture has become common condition, and until today, the treatments available are not satisfactory.[14]

As potent therapies become increasingly available in osteoporosis and metabolic bone diseases, there is also growing interest in the pharmacological application of fracture repair.[31] A number of well-known anti-resorptive agents have been explored to inhibit excessive bone resorption and formation while increasing bone density generally exists. For example; bisphosphonates, estrogens, selective estrogen-receptor modulators, calcitonin, vitamin D, and calcitriol have been adopted to treatment for postmenopausal women with osteoporosis.[22] In addition, human parathyroid

Address correspondence to Eiji Tanaka, Department of Orthodontics and Dentofacial Orthopedics, Institute of Health Biosciences, Tokushima University Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan. Electronic mail: etanaka@tokushima-u.ac.jp

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(PTH), when introduced intermittently, has been shown to have an efficacious anabolic effect leading to an increase of bone mass and improvement of bone architecture.[23] Furthermore, it also has been addressed to enhance bone mass in severe osteoporotic humans and in postmenopausal women,[6,20,37] young and old rats, normal, as well as ovariectomized (OVX) rats.[15]

Meanwhile, consistent mechanical stresses have been known to activate both osteoblast and osteoclast, which are important for the enhancement of bone mass and strength.[3] Numerous types of mechanical stimuli have been shown to promote proliferation and differentiation of osteoblast. Among them, low-intensity pulsed ultrasound (LIPUS) has demonstrated to be very efficient in this regard[8]; LIPUS also served as an anabolic agent during fracture healing. LIPUS is a form of mechanical energy that can be transferred into living tissue as high frequency acoustical pressure waves, resulting in biochemical events at the cellular level.[1,34] It has been frequently reported that the application of LIPUS in animal fracture models improved the restoration of mechanical strength of intact bone by 30–38%.[33] LIPUS has also been known to accelerate bone maturation in distraction osteogenesis in rabbits, even in states of poor callotasis.[27] In addition, LIPUS stimulation could accelerate the osteoporotic fracture healing *via* faster response of endochondral ossification and greater increase of bone mechanical property.[7,30]

Satisfactory healing of osteoporotic fracture is very essential for functional recovery, morbidity and quality of life. While there is developing fact to support the individual application of PTH and LIPUS during osteoporotic fracture healing,[7,17,18,35] their effects have not yet been reported when introduced concurrently. The aim of this study is, thus far, to investigate the effects of combined PTH (1–34) and LIPUS on fracture healing in OVX rats.

MATERIALS AND METHODS

Animal Preparation

The care and experimental protocol of this study were approved by the Animal Care and Use Committee of Tokushima University. Thirty-two Sprague–Dawley female rats, 12-week-old, weighing 220–260 g (Nihon SLC Co., Shizuoka, Japan), were fed on conventional solid diet (CLEA Japan, Tokyo, Japan) and water ad libitum. To induce bone loss, all the animals were OVX, and lumbar vertebrae of each animals were scanned. After 3 months of OVX, the lumbar vertebras were rescanned to confirm the changes in microarchitectures (67.0% decrease in bone volume fraction [BV/TV] and 24.2% decrease in trabecular bone thickness with OVX, data not shown). Experimental animals were randomly allocated to 4 groups: control group as placebo, PTH group, LIPUS group, and combined group ($n = 8$ for each group).

Fracture Induction

All OVX rats experienced surgery to produce bilateral mid-diaphyseal fractures of proximal tibiae.[36] The animals were anesthetized through the intra-abdominal injections of 50-mg/kg pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). In a sterile condition, we created medial para-patellar incision and laterally dislocated the patella. A wire cutter was used for creating tibial fracture at their midshaft. Then, a 0.032-inch Titanium-Molybdenum alloy wire (TMA, Ormco Co., Orange, CA) was inserted to the canal of intramedullary tibia, and followed by drilling and reaming it with a round bar.

LIPUS Application

We used LIPUS exposure system that is modified from the clinical device (Osteotron D2, Ito Co., Tokyo, Japan). The ultrasound exposure system was equipped with a circular transducer of 18 mm in diameter. The ultrasound head of this device had a beam non-uniformity average of 3.6 and effective radiating area of 0.9 cm^2 (36%). A pulsed ultrasound signal was transmitted at a frequency of 1.5 MHz with a spatially averaged intensity of 30 mW/cm^2 and pulsed 1:4 (2 ms on and 8 ms off). The fracture sites received LIPUS 5 days/week for 20 min/day. LIPUS and combined groups receive LIPUS exposure for the lasting 6 weeks.

PTH Administration

PTH [synthesized human PTH (1–34)] was provided from Asahi Kasei Pharma Corporation (Tokyo, Japan). PTH was dissolved in a physiologic saline solution containing 0.1% rat serum albumin (RSA). PTH and combined groups were administered PTH subcutaneously in the dorsal region at a dose of 30 μg/kg/day for 3 days/week for 6 weeks. The 30-μg/kg/day dose was chosen on the basis of previous fracture-healing studies showing significant beneficial effect at this dose.[2,28]

Radiography and Micro-Computed Tomography

Fracture healing was monitored weekly on anteroposterior radiography and Micro-CT (μCT) through the experimental period. For all animals, radiography and μCT were collected immediately after fracture and

at weekly intervals for 6 weeks ($n = 5$ for each group). From radiography, fracture healing at each week was evaluated as bridged if the cortex of the bone is observed as bridging. The fracture healing at each week was assessed using a 4-point radiographic scoring system by Warden *et al.*:[32] 0 = no evidence of healing; 1 = callus formation evident but fracture gap not yet bridged; 2 = callus formation evident with possible bridging of the fracture gap; 3 = fracture union. Furthermore, the radiographs were scanned by a flat-bed scanner, and copied into imageJ 1.48r (National Institute of Health, Bethesda, MD). All callus detected beyond the bone's original periosteal surface was traced, and projected callus area (mm^2) was measured. Bone bridging and callus area determination were evaluated by two examiners, who were unaware of 4 groups.

The μCT was used to assess fracture site bone mineral density (BMD; mg/cm^3) and structural parameters of trabecular bone including trabecular number (Tb.N; mm^{-1}), trabecular thickness (Tb.Th; μm), and bone volume fraction (BV/TV; %). Each tibia was blindly scanned using the *in vivo* μCT scanner for small animal lab *Latheta* LCT-200 (Hitachi *Aloka* Medical, Tokyo, Japan). We implemented standard three-dimensional measures of trabecular architecture as plug-in, BoneJ for imageJ.[10] Anesthetized rats were placed prone on a bed made by polystyrene form with a scanning condition of pixel counts: 512 × 512, effective field of view: 120 mm, pixel size: 60 × 480 μm, slice thickness: 480 μm, and rotate angle: 360° acquisition. Region of interest (ROI) was 3.5 mm proximal and distal to the fracture line with 7 mm in total.

Histometrical Analysis

To investigate the effect of treatments on fracture healing histologically, 5 weeks after the fracture ($n = 3$ each group), the animals were anesthetized with 50 mg/kg pentobarbital following sacrificed by perfusion through the left ventricle with PBS for 30 s, followed by a fixative solution consisting of 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) and 0.1% glutaraldehyde (Katayama Chemical, Osaka, Japan) in 0.08 M sodium cacodylate (Wako Pure Chemical Industries) buffer consist of 0.05% calcium chloride (Junsei Chemical, Tokyo, Japan), pH 7.2, for 20 min. Tibiae were removed and samples were soaked in the same fixative solution overnight at 4°C. After fixation, the tibiae were rinsed in sterile phosphate buffered saline solution and decalcification was pulled of with use of 10% EDTA (pH 7.0, Life Technologies, Frederick, MD) for 2 weeks on a shaker at 4°C. EDTA solution was changed every 2 days. The tibiae were cut into halves ahead the mid-sagittal plane. The samples were embedded into paraffin and sectioned in 5 μm of thickness and they were mounted onto the silane-coated glass slides and stained with Hematoxylin Eosin (HE) following by examination by a microscope.

Mechanical Testing

Mechanical properties of the fracture site were determined by testing the tibiae in three-point bending apparatus. Three-point bending test was also selected in the present study, because it was convenient, efficient and possible to locate the loading bar at the fracture site to test the specific part of the bone by using the three-point bending test.[12] The test was performed 6 weeks after fracture induction ($n = 5$ for each). A universal testing machine (Shimadzu AG-X, Shimadzu, Kyoto, Japan) was used to test the tibiae to failure with a constant displacement rate at 10 mm/min. The tibia were loaded in an anteroposterior direction with a span length of 13 mm. Bending moment to failure (M) was calculated as ¼ × breaking load (N) × span (13 mm).[9]

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). For the mechanical testing, differences between groups were tested by Student's t test. Comparisons among 4 groups were performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test. In all cases, differences were considered statistically significant at the 5% level of significance with the Statistical Package for the Social Sciences (SPSS version 22; Chicago IL).

RESULTS

Radiography of the Fractured Tibiae in OVX Rats

Figure 1 shows representative longitudinal radiographic images of bilateral mid-diaphyseal tibial fractures at different time points. For all the groups, a callus formation was detected within 2 weeks, and increased in size during the succeeding 1–2 weeks. The fracture line on the original bone disappeared by weeks 6, 5, and 4 in LIPUS, PTH, and combined groups, respectively. In control group, the fracture line still remained at week 6.

For radiographic scoring of fracture healing, the fracture healing score was significantly lower ($p < 0.01$) in the control group than in the remaining 3 groups. There was also a significant difference

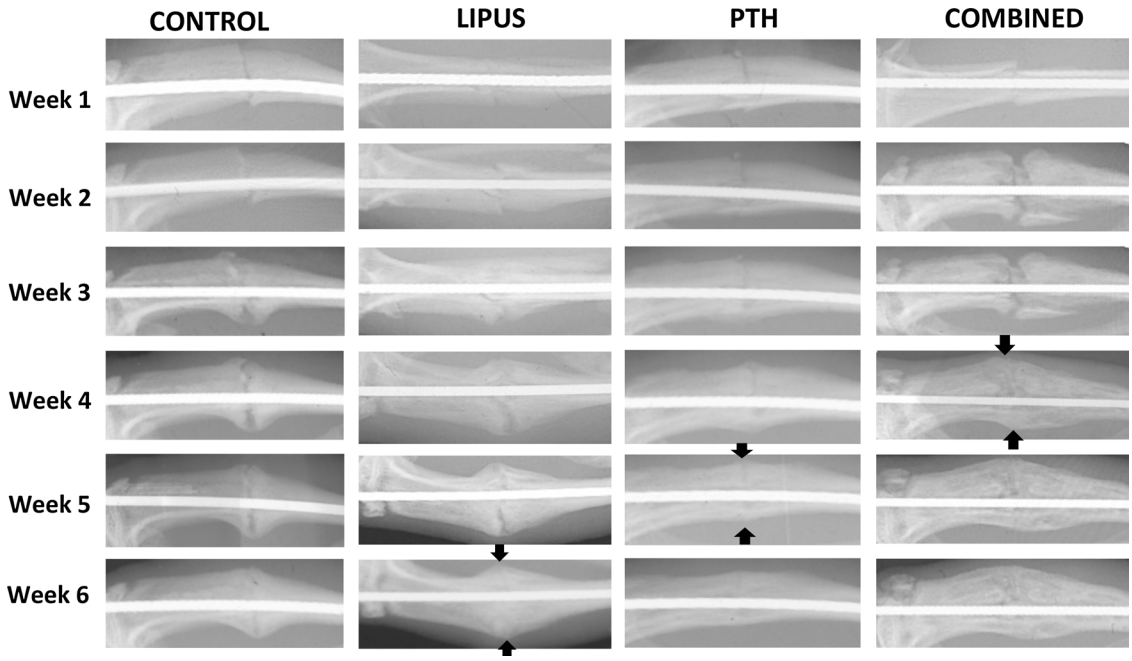

FIGURE 1. Representative longitudinal radiographic images of mid-diaphyseal tibial fractures in 4 groups. Combined group showed earliest fracture gap bridging at week 4, compared to the remaining 3 groups. Arrows indicate the initial occurrence of callus bridging.

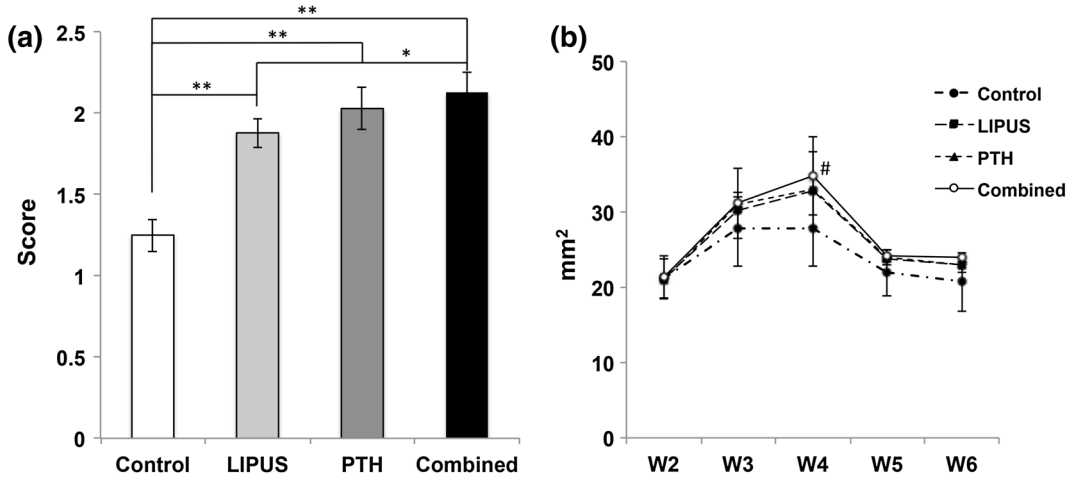

FIGURE 2. Radiographic analysis of bilateral tibial fracture. (a) The fracture healing score was significantly lower in control group than in the remaining 3 groups. *$p < 0.05$, **$p < 0.01$. (b) Mean callus area. Significant difference was found between control and combined group (#) at week 4 ($p < 0.05$).

($p < 0.05$) in the radiographic scores between combined and LIPUS groups (Fig. 2a).

For projected callus area (mm^2), the control group showed weaker callus formation through weeks 3–6, compared to the remaining 3 groups (Fig. 2b). At week 4, there was a significant difference ($p < 0.05$) in the mean callus area between control and combined groups.

μCT of the Fractured Tibiae in OVX Rats

Control group had no or less changes in the measurements of BMD, Tb.N, Tb.TH, and BV/TV through the experiment period. Although LIPUS group also revealed relatively small changes in the measurements, most of the measurements in LIPUS group were larger than those in control group. For

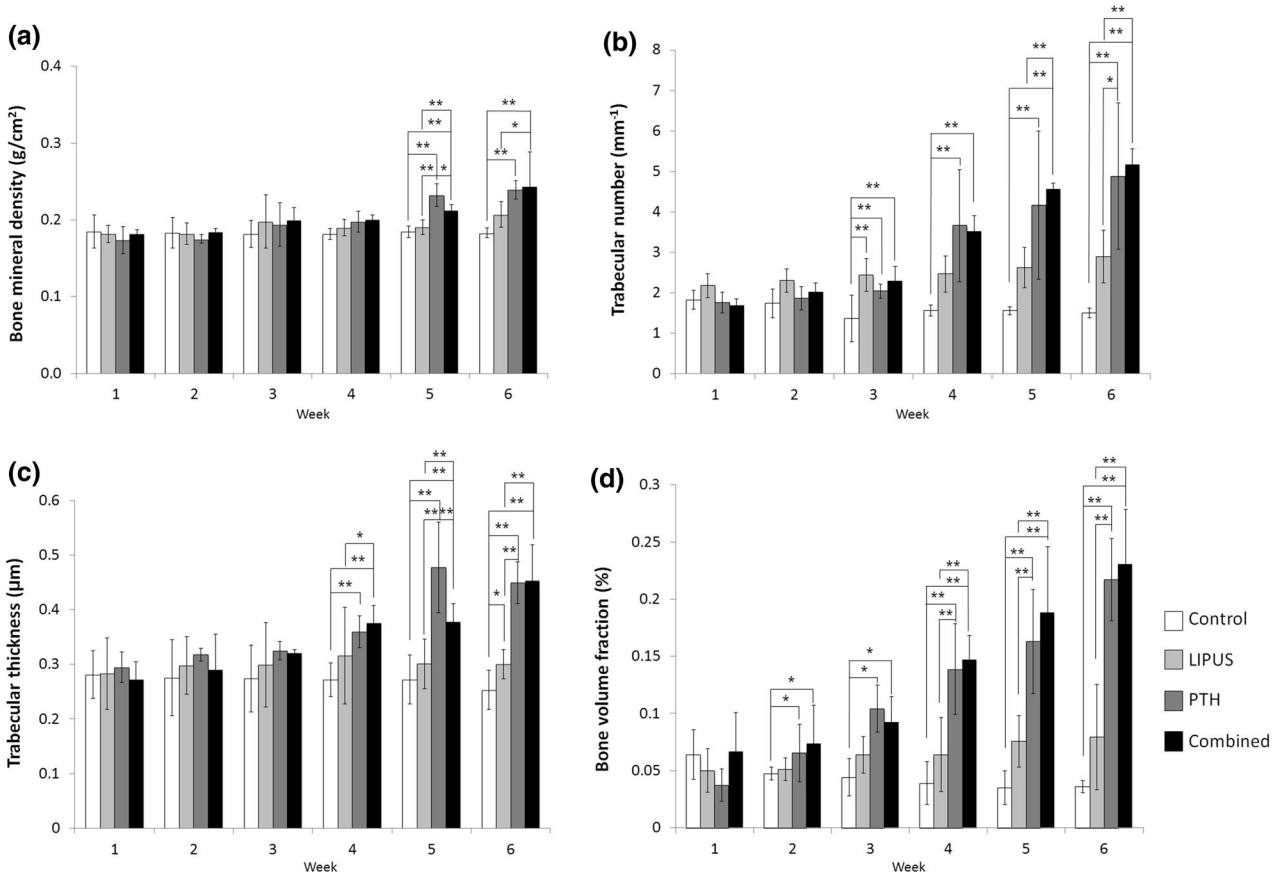

FIGURE 3. Effects of PTH, LIPUS and their combination on μCT outcomes during osteoporotic fracture healing. (a) BMD. At weeks 5 and 6, PTH and combined groups revealed significantly higher BMD compared to control and LIPUS groups. (b) Tb.N. PTH and combined groups had significantly greater Tb.N than control group at weeks 3–6. (c) Tb.TH. Tb.TH in PTH and combined groups were significantly greater than control and LIPUS groups at weeks 4–6 and weeks 5–6, respectively. (d) BV/TV. At weeks 2–6, BV/TV value was significantly higher in PTH and combined groups than in control group. Data are mean followed by SE with significant differences from control, LIPUS, PTH, or combined group. *$p < 0.05$, **$p < 0.01$.

BMD, PTH and combined groups showed an increase at week 3, and remained a higher value than control group thereafter. At week 5, PTH group had a significantly higher BMD ($p < 0.05$ or $p < 0.01$) compared to the remaining groups. The combined group had also a higher BMD compared to the control and LIPUS groups at weeks 5 and 6, and had the highest BMD at 6 week (Fig. 3a).

Tb.N was larger in LIPUS group than in control group, and a significant difference ($p < 0.01$) between LIPUS and control groups was found at week 3. PTH and combined groups had significantly greater Tb.N ($p < 0.01$) than control group at weeks 3–6 (Fig. 3b). Tb.TH in PTH and combined groups increased at 3-week post fracture and was significantly greater ($p < 0.01$) than control and LIPUS groups at weeks 4–6 and weeks 5–6, respectively. LIPUS group also showed a larger Tb.TH compared to control group and a significant difference ($p < 0.05$) was found between control and LIPUS groups only at week 6 (Fig. 3c). From week 2, a marked increase in BV/TV values was observed through the experimental period in PTH and combined groups. At weeks 2–6, BV/TV value was significantly higher ($p < 0.05$ or $p < 0.01$) in PTH and combined groups than in control group. BV/TV value in LIPUS group was kept larger than that in the control group; however, compared to PTH and combined groups, significantly smaller values ($p < 0.01$) were found at weeks 4–6. Furthermore, the combined group revealed a higher ratio of increment in BV/TV value compared to the remaining 3 groups during weeks 3–6 (Fig. 3d).

Histology

Figure 4 shows representative histological images of proximal tibial fracture in 4 groups at 5 weeks post fracture. In control group, abundant chondroid tissues

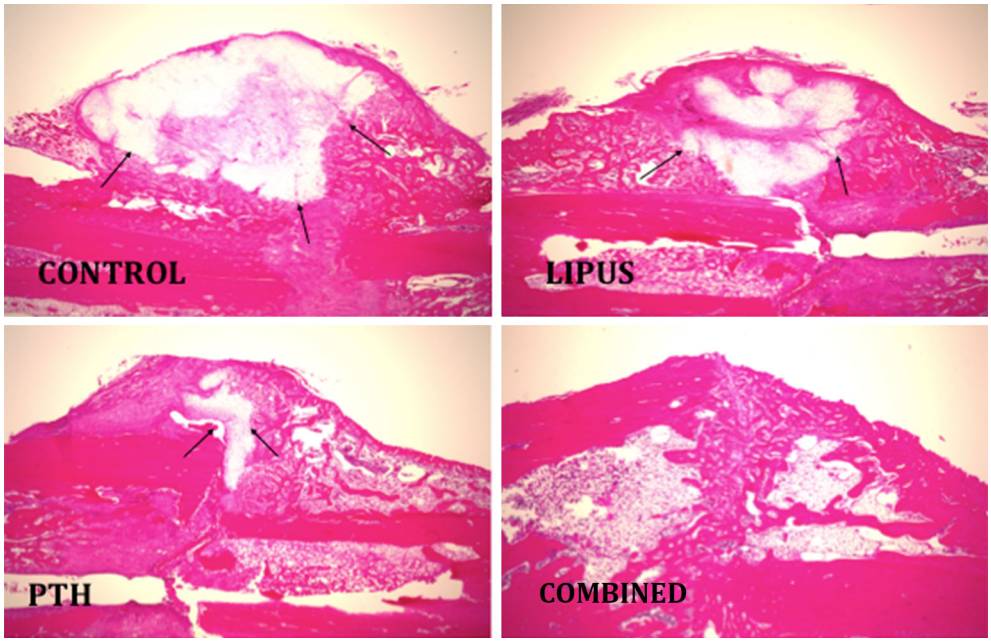

FIGURE 4. Representative histological images of midshaft tibial fractures at 5 weeks post fracture. The amount of chondroid tissues was reduced in PTH and LIPUS groups and slightly replaced by osseous tissues (indicate by arrows). Combined group showed fracture line already replaced by osseous tissues.

filling the fracture site indicated the continuation of active endochondral ossification through the 5-week post treatment period, while in PTH and LIPUS groups, the amount of chondroid tissues was reduced and slightly replaced by osseous tissues. The combined group showed a complete replacement of fracture line by osseous tissues.

Mechanical Testing

Bending moment to failure in the control group was 166.4 ± 25.4 N-mm, which was the smallest in 4 groups (Fig. 5). Combined group had the greatest bending moment (420.8 ± 68.8 N-mm), followed by PTH and LIPUS groups (401.2 ± 112.5 N-mm and 293.4 ± 162.9 N-mm, respectively). The value of bending moment to failure was significantly ($p < 0.05$ or $p < 0.01$) smaller in the control group than in the remaining 3 groups.

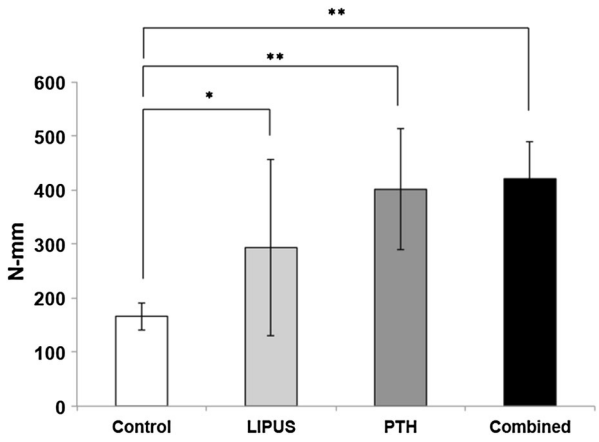

FIGURE 5. Biomechanical testing of fracture site. Combined group had the greatest bending moment, followed by PTH and LIPUS groups. The value of bending moment to failure was significantly smaller in control group than in the remaining 3 groups. *$p < 0.05$, **$p < 0.01$.

DISCUSSION

Although systemic agents such as bisphosphonate (BP) and PTH have an effectiveness to reduce the risk of certain fractures up to 80%,[4,21] their systemic administration may not be sufficient to effectively treat all cases of osteoporosis and their treatment duration may be limited. In addition, as with any medication, there are risks and side effects involved with osteoporosis treatments. For example, extended BP treatment has been associated with abnormal subtrochanteric fractures and osteonecrosis of the jaw.[5,25,26] Furthermore, limited drug bioavailability may prolong healing of existing fractures, especially in previously untreated bone, by not achieving an effective local dose.[19] Since satisfactory healing of the osteoporotic fracture is essential to life, management of osteoporotic fracture and prevention of repeated osteoporotic fracture remain difficult clinical chal-

lenge. Then, an alternative local treatment strategy without deteriorative side-effects is indispensable for enhancing osteoporotic fracture healing by systemic agents.

In vivo studies have demonstrated that LIPUS can promote tissue repair and regeneration, enhance bone formation at the sites of distraction osteogenesis and accelerate bone fracture healing.[1,11,13] It is generally accepted that LIPUS has no deleterious or carcinogenic effects. In addition, LIPUS exposure has no thermal effects to produce biological changes in living tissues. Due to its non-invasive nature, LIPUS has a potential to be used as an additive therapy for each clinical setting. After Heckman *et al.*[13] first showed LIPUS accelerated fracture healing in humans, LIPUS has been studied for the local treatment and prevention of osteoporotic fracture.[13,35] However, its effectiveness remained controversial in certain respects. Our results from weekly radiography, histomorphometry, μCT, and mechanical test showed LIPUS treated group was with better healing responses than control group. Furthermore, LIPUS group revealed higher callus size at 4-week post fracture compared to control group. Meanwhile, PTH group showed similar callus formation, earlier callus bridging and earlier replacement of fracture line by osseous tissues than LIPUS group. These are consistent with a previous report that LIPUS primarily increases callus size without changes in BMD, while PTH administration has the opposite effect of increasing callus BMD without influencing in size.[34] However, the effectiveness of LIPUS on osteoporotic fracture healing was relatively lower compared to systemic administration of PTH. This implies that LIPUS alone could not accomplish satisfactory healing of osteoporotic fracture.

To our knowledge, the effect of the combined therapy on osteoporotic fracture has not previously been investigated. Correspondingly, the present study would be the first to investigate the potential of PTH, LIPUS and their combination for healing osteoporotic fracture from biological and biomechanical perspective in OVX model rats. It is well recognized that PTH increases BMD and reduces the risk of fracture mainly by stimulating bone formation, while LIPUS primarily increases callus size without changes in BMD.[17,34] Therefore, the logical question was whether joint use of these therapies would provide therapeutic advantage or not. In our result, although the combined therapy of PTH and LIPUS has no additive effects on osteoporotic fracture healing, both PTH and LIPUS enhanced osteoporotic fracture healing and the beneficial PTH effect might not be impaired by the LIPUS exposure to osteoporotic fracture site.

Previous study by Warden *et al.*[34] in which the individual and combined effects of PTH and LIPUS on normal fracture healing were investigated. The report indicated that the effect of LIPUS in the combined therapy during osteoporotic fracture healing was additive but not synergistic. Furthermore, in our results, the combined group was with better healing processes than individual PTH and LIPUS groups and revealed a higher ratio of increment in BV/TV value compared to PTH group during weeks 3–6. Therefore, it is suggested that LIPUS, as a local treatment strategy in combination with systemic administration of PTH, may prove effective to promote local bone healing in osteoporotic fracture.

It is demonstrated that the risk of osteoporotic bone fracture is related to a decrease in the mechanical properties of bone induced by osteoporosis.[7,18] In other words, the enhancement of mechanical strength and stiffness in osteoporotic fracture site is critically important for preventing secondary fractures in bedridden patients. Our results showed that the combined group of PTH and LIPUS had the highest bending moment to failure at fracture site compared to the LIPUS or PTH treated group. This may result from the beneficial effects of the combination wherein LIPUS first accelerates callus formation and PTH induces replacement of fracture line by osseous tissues thereafter.

In conclusion, our results demonstrated that the combined therapy of PTH and LIPUS leads to accelerated fracture healing and enhanced bone properties through improved bone volume and microarchitectural parameters compared to the individual treatment. These data suggest that the combined treatment of PTH and LIPUS might be beneficial in osteoporotic fracture healing. Moreover, further investigation of the effects of PTH administered with different manner in combination with LIPUS should be tested.

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DISCLOSURE

All authors state that they have no conflicts of interest.

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