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Research Article

Effects of Alendronate and Low-Intensity Pulsed Ultrasound Therapies at Osteoporotic Cancellous Osteotomy Sites in Proximal Tibia of Ovariectomized Rats

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Abstract

Introduction: The effects of Alendronate (ALN) and Low-Intensity Pulsed Ultrasound (LIPUS) on cancellous bone repair under osteoporotic conditionsremain unclear. The aim of the present study was to evaluate the effects of ALN and/or LIPUS on cancellous bone repair at cancellous bone osteotomy sites in Ovariectomized (OVX) rats.

Materials and Methods: Seven-month-old female Sprague-Dawley rats were subjected to osteotomy at the proximal tibial metaphysis 4 weeks after OVX. The rats were randomized into the following 4 groups: 1) Control group, treated with ALN vehicle and sham LIPUS; 2) ALN group, treated with ALN (1 μg/kg/day) and sham LIPUS; 3) LIPUS group, treated with ALN vehicle and LIPUS (20 min/day); and 4) ALN+LIPUS group, treated with ALN and LIPUS. Bone Mineral Density (BMD), bone histomorphometry, cancellous bone union rate, and bone strength at the cancellous bone osteotomy site were assessed after 2or4 weeks of treatment.

Results: Two weeks of ALN, LIPUS, and combined treatment significantly increased bone volume (BV/TV) ($p < 0.001$, $p = 0.012$, and $p = 0.0014$, respectively). ALN and combined treatment for 2 weeks significantly increased the ultimate load ($p = 0.029$ and $p = 0.048$, respectively) and breaking energy ($p = 0.010$ and $p = 0.019$, respectively) compared with those of control. Four weeks of ALN treatment increased BMD ($p <0.05$), BV/TV ($p = 0.029$), but not cancellous bone union rate and bone strength, compared with control. LIPUS therapy for 4 weeks did not increase the BMD, BV/TV, bone union rate, and bone strength at the osteotomy site. Combined treatment with ALN and LIPUS produced asignificant increase in BMD ($p < 0.01$), BT/TV ($p = 0.029$), and cancellous bone union rate ($p = 0.048$), compared with control at 4 weeks.

Conclusion: Combined therapy with ALN and LIPUS increased cancellous bone repair and bone strength in OVX rats.

Keywords: Bisphosphonate; Low-intensity pulsed ultrasound; Cancellous bone repair

Introduction

Osteoporosis is a major health problem because fractures caused by osteoporosis lead to decreased Quality Of Life (QOL) and increasedpatient mortality [1]. Fragility generally arisesfromdecreased bone mass and/or quality in cortical bone and cancellous bone, and fragile fractures are likely to occur in regions rich in cancellous bone such as vertebral body or proximal femur. These fractures are critical fracture to determine the QOL or mortality of patients [1]. Furthermore, the first fragility fracture is a very strong predictor for additional fractures. Thus, a treatment for osteoporosis should be started as soon as possible during a treatment or operation for fragility osteoporotic fractures to prevent these secondary fractures.

However, it has been reported that pharmacological therapy for osteoporosis following fragility fractures are not performed adequately [2,3]. One of reasons of the low proportion of treatment for osteoporosis after a fracture is the fear of pharmacological

agents for osteoporosis may negatively interfere with fracture healing [4,5]. Although one of Bisphosphonates (BPs), Alendronate (ALN) is broadly usedto preventbone fragility and the incidence of fractures in postmenopausal women [6,7], ALN might inhibit fracture healing because of its effect of suppressing bone turnover. Fu et al. suggested that ALN was beneficial for the mechanical properties of cortical bone, however it delayed cortical bone remodeling in Ovariectomized (OVX) rats [8]. Other studies have demonstrated that BPs result in the formation of a stronger and denser callus with no delay on fracture healing in cortical bones in animal models [9-12]. However, osteoporotic fragility fractures often occur at cancellous bone rich sites, such as in the vertebral body or proximal femur. Effects of ALN on cancellous bone healing under osteoporotic condition are not fully elucidated.

To resolve bone remodeling and healing, Low-Intensity Pulsed Ultrasound (LIPUS) is a clinically available modality for accelerating fracture healing [13]. LIPUS is able to lessen the time to union of fresh fracturesby > 30% [14,15], and is also beneficial in situations that are disadvantageous for healing [16]. If ALN may negatively interfere with cancellous bone healing, combined treatment with ALN and LIPUS could improve conditions at cancellous fragile fracture sites more than ALN mono-therapy.

We have reported that the combined therapy of ALN and LIPUS did not affect cancellous bone union in aged rats [17]. However, the effects of ALN and/or LIPUS on cancellous bone healing under osteoporotic conditions caused by OVX remain unclear. The purpose of this study was to evaluate the effects of ALN and/or LIPUS on cancellous bone volume, bone union, and bone strength at bone osteotomy sites at the proximal tibial metaphysis in OVX rats.

Materials and Methods

Animals

Six-month-old female Sprague-Dawley rats (SLC, Tokyo, Japan) were housed in a controlled environment at 22^{0}C with a 12-h light/dark cycle. Rats were pair-fed and allowed ad libitum access to water and standard diet (CE-2; Clea Japan Inc., Tokyo, Japan) containing 1.14% calcium, 1.06% phosphorus, and 250 IU vitamin D3 per 100 g of food, as described previously [18,19]. The osteoporosis model was induced by bilateral OVX at six months of age followed by four weeks of rearing [20]. The establishment of post-menopausal osteoporosis was confirmed by the presence of uterine atrophy.

Cancellous bone osteotomy in the right proximal tibia

Cancellous bone osteotomy was performed on the right proximal tibia of each rat at seven months of age. A median parapatellar incision from the knee joint through the proximal half of the tibia was made on the right hind limb. Using an electric bone saw (Yoshida Medical Inc., Ehime, Japan), an incomplete mid-sagittal osteotomy was created from the joint surface around one-quarter of the proximal tibia, without extending to the caudal cortex [21]. The osteotomized tibia was closed using a non-absorbable suture. After surgery, the rats were allowed to move freely. No animal was observed with abnormal gait or impaired locomotion postoperatively.

Treatment protocol

The rats were randomized into four groups (n = 20 each group): (1) Control group (OVX rats treated with saline (ALN vehicle) and sham-LIPUS); (2) LIPUS group (OVX rats treated with ALN vehicle and LIPUS radiation); (3) ALN group (OVX rats treated with ALN and sham-LIPUS); and (4) ALN+LIPUS group (OVX rats treated with ALN and LIPUS radiation). These treatments were conducted under intraperitoneal anesthesia and initiated two dayspost-osteotomy and continued until sacrificing at two or four weeks later (n = 10 each). The rats were euthanized with anintra-peritoneal injection of ketamine hydrochloride (20 mg/kg) (Ketalar; Daiichi Sankyo Propharma, Tokyo, Japan) and xylazine hydrochloride (1.5 mg/kg) (Sederac; Nippon Zenyaku Kogyo, Fukushima, Japan). The right tibia were harvested and fixed in 10% neutral-buffered formalin.The right femur was also harvested with muscle and frozen under -80^{0}C. All animal experiments were approved by our institute and conducted according to the "guidelines for animal experimentation". This study was started at May 2013 and ended at August 2015.

Ultrasound intervention

The LIPUS apparatus was supplied by Sonic Accelerated Fracture Healing Systems (SAFHS®4000J; Teijin Pharma, Tokyo, Japan). LIPUS is often utilized to accelerate bone union. The efficacy of LIPUS has beenpreviouslydemonstratedin animal models [13,22,23]. The signal strength and duration of treatment used in this study were in accordance with conditions endorsed for clinical situations. The ultrasound signal generated with a transducer consisted of a burst width of 200 μs containing 1.5 MHz sine waves repeated at a frequency of 1.0 kHz, and a Spatial Average-Temporal Average (SATA) intensity of 30 mW/cm^2 for 20 minutes per day. Animals were anesthetized by an intra-peritoneal injection of a mixture ketamine (20 mg/kg) and xylazine (1.5 mg/kg) during LIPUS or sham radiation. The gel and elasticband of this device were attached in a manner that ensured radiation was delivered to the osteotomy site (Figure 1).

Figure 1: LIPUS exposure.

LIPUS radiation delivered to the osteotomy site of the right proximal tibia was performed under anesthesia, and the transducer was attached with an elastic band.

Alendronate administration

An ALN (Wako Junyaku, Osaka, Japan) solution was prepared in saline at a concentration of 0.02 mg/mL. Rats in the ALNand ALN+LIPUS groups received a daily subcutaneous injection of ALN (1 μg/kg). This dosage of ALNwas equivalent to the dosage used in humans (5 mg/day, peroral) and chosen to be in accordance with that used in previous animal studies [23-25]. Saline was selected as the vehicle control, and 0.2 mL of saline was administered as a subcutaneous injection to rats in the control and LIPUS groups. Body weights were measured weekly and injection dosages were adjusted accordingly.

Assessment

Measurement of Bone Mineral Density (BMD)

BMD was measured by dual-energy X-ray absorptiometry (DXA, Hologic QDR-4500, Hologic, MA, USA) in an anteroposterior plane. We assessed BMD of the right proximal tibia at the osteotomy site. The region of interest was 20 mm and one-third of the tibial length from the proximal edge of the tibia. Two scans of each sample were performed and average values of BMD were used for analysis [23].

Sample preparation

After BMD measurements, the proximal half of the right tibia, including the osteotomy site, were decalcified with neutral ethylene diamine tetra acetic acid for four weeks and embedded in paraffin. Subsequently, 3-micron-thick mid-frontal sections were prepared for hematoxylin and eosin (H-E) staining to assess cancellous bone histomorphometry and to evaluate bone union.

Bone histomorphometry and bone union rate

Bone histomorphometrical analyses of H-E stained sections were performed with a semiautomatic graphic system (Histometry RT CAMERA, System Supply Co., Nagano, Japan). Measurements were obtained in regions 390 μm from the lowest point of the growth plate-metaphyseal junctions in the caudal direction, to exclude the primary spongiosa, as well as 390 μm from the endocortical surfaces [17,26]. The cancellous bone volume per tissue volume (BV/TV), Osteoid Surface per Bone Surface (OS/BS), Eroded Surface per Bone Surface (ES/BS) and osteoclast number per bone surface (Oc.N/BS) were measured at a magnification of 200 [27]. The bone union rate was evaluated at 100 magnification within the same field as histomorphometry. We defined cartilaginous bonding as bony union, while fibrous assimilation as nonunion. The proportion of bony union in the total length of the osteotomy line was counted [17,21,26].

Mechanical properties of cancellous bone

Because the area subjected to LIPUS radiation included the distal metaphysis of the femur, we performed compression tests to assess the mechanical properties of the distal femur. The distal metaphysis of the femur was cut using an electric saw 10 mm from the joint surface of the condyle. A compression load was applied to the specimens using a rectangular parallelepiped crosshead (length 20 mm, width 20 mm, and height 10 mm) from the lateral aspect to the medial aspect. Load and displacement curves were recorded at a crosshead speed of 10 mm/min and compressive depth up to 2.5 mm, and the following extrinsic parameters were calculated by the testing machine software (CTR win; System Supply Co., Nagano, Japan): ultimate load (N), stiffness (N/mm), and breaking energy (Nm) [28,29].

Statistical analyses

All data are expressed as mean ±Standard Deviation (SD). Differences between groups at each time point were evaluated using One-Way Analysis Of Variance (ANOVA). Multiple comparisons were made using either Scheffe's or Friedman's post hoc tests, as appropriate. Two-factor factorial ANOVA was performed to evaluate the effect of ALN or LIPUS alone and the interaction between these interventions. All statistical analyses were performed using the Statistical Package for the Biosciences software (SPBS v 9.6) [30]. Probability values of less than 0.05 were considered statistically significant.

Results

BMD (Table 1):

BMD (mg/cm^2)	Control	ALN	LIPUS	ALN+LIPUS	Two factor factorial ANOVA		
					ALN	LIPUS	Interaction
2 wks	0.225 ± 0.012	0.245 ± 0.013	0.229 ± 0.013	0.250 ± 0.023	p = 0.068	p = 0.58	N.S.
4 wks	0.229 ± 0.022	0.260 ± 0.011*	0.241 ± 0.012*	0.275 ± 0.026	p < 0.001	p = 0.12	N.S.

All values are mean ± SD
Control, Saline + Sham LIPUS; ALN, Alendronate + Sham LIPUS; LIPUS, Saline + LIPUS; Combined, Alendronate + LIPUS
N.S., not significant; *, $p < 0.05$ vs. control group; †, $p < 0.01$ vs. control group; ‡, $p < 0.05$ vs. LIPUS group by one-way ANOVA with the Friedman post hoc test.

Table 1: Bone mineral density (BMD) at the osteotomy site of proximal tibia.

Aftertwo weeks (six weeks after OVX) of ALN, LIPUS, and ALN+LIPUS treatment, no effect on BMD was observed. ALN administration for four weeks significantly increased BMD (without interaction; $p < 0.001$; two-factor factorial ANOVA). The BMD of the ALN ($p < 0.05$), LIPUS ($p < 0.05$) and ALN+LIPUS ($p < 0.01$) groups were significantly higher than that of control group,according to the Friedman post hoc test. The BMD of the ALN+LIPUS group was also significantly higher ($p < 0.05$) than that of the LIPUS group after four weeks of treatment.

	Control	ALN	LIPUS	ALN+LIPUS		Two factor factorial ANOVA		
						ALN	LIPUS	Interaction
2 wks								
BV/TV (%)	16.05 ± 1.61	26.18 ± 9.00	21.10 ± 4.30	34.36 ± 4.68[a,b]		p < 0.001	p = 0.012	N.S.
OS/BS (%)	49.03 ± 3.83	50.15 ± 1.64	52.24 ± 6.37	55.89 ± 5.00		p = 0.32	p = 0.066	N.S.
ES/BS (%)	28.83 ± 3.32	26.72 ± 3.43	31.49 ± 2.58	27.12 ± 3.86		p = 0.063	p = 0.38	N.S.
Oc.N/BS (N/mm)	4.1 ± 0.3	2.8 ± 0.9[c]	3.8 ± 0.8	2.6 ± 1.1[d]		p = 0.001	p = 0.37	N.S.
4 wks								
BV/TV (%)	17.93 ± 6.96	34.42 ± 10.75[e]	24.04 ± 4.53	34.50 ± 8.93[e]		p = 0.002	p = 0.43	N.S.
OS/BS (%)	63.73 ± 3.18	60.90 ± 4.90	58.16 ± 8.54	63.80 ± 6.31		p = 0.53	p = 0.58	N.S.
ES/BS (%)	27.28 ± 3.93	22.82 ± 7.39	25.17 ± 6.12	21.92 ± 5.78		p = 0.052	p = 0.46	N.S.
Oc.N/BS (N/mm)	4.1 ± 1.6	2.9 ± 0.8	3.9 ± 1.1	2.7 ± 1.0[f]		p = 0.002	p = 0.40	N.S.

All values are mean ± SD
Cancellous bone volume per tissue volume (BV/TV); osteoid surface per bone surface (OS/BS); eroded surface per bone surface (ES/BS); osteoclast number per bone surface (Oc.N/BS)
a, p = 0.0014 vs. control group; b, p = 0.015 vs. LIPUS group; c, p = 0.002vs. control group; d, p = 0.001 vs. control group; e, p = 0.029 vs. control group; f, p = 0.030 vs. control group by one-way ANOVAwiththe Scheffe post hoc test.

Table 2: Bone histomorphometric indices at the osteotomy site of proximal tibia.

The analysis showed that ALN treatment significantly increased BV/TV ($p < 0.001$ and $p = 0.002$, respectively) and decreased Oc.N/BS ($p = 0.001$ and $p = 0.002$, respectively) at two and four weeks by two factor factorial ANOVA. In comparison, LIPUS significantly increased BT/TV ($p = 0.012$) at two weeks.

At two weeks, the BV/TV of the ALN+LIPUS treatment group was significantly higher than the control ($p = 0.0014$) and LIPUS ($p = 0.015$) groups. The Oc. N/BS of the ALN and ALN+LIPUS groups were significantly lower than the control group ($p = 0.002$ and $p = 0.001$, respectively).

At four weeks of treatment, the BV/TV of the ALN and ALN+LIPUS groups were significantly higher than the control group ($p = 0.029$ and $p = 0.029$, respectively). Additionally, the Oc.N/BS of the ALN+LIPUS group was significantly lower than the control group ($p = 0.030$).

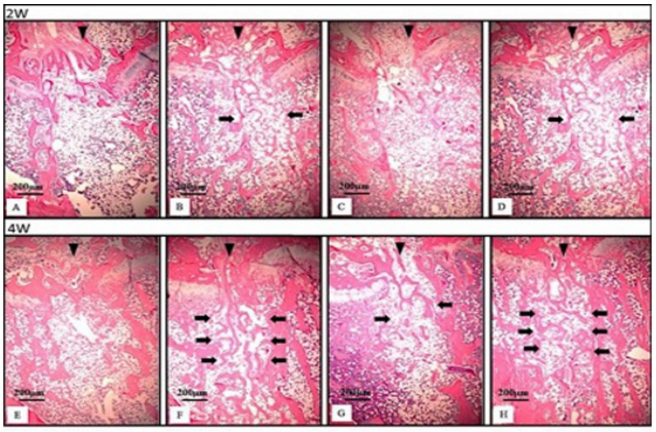

Figure 2: Histological samples of the osteotomy site stained with Hematoxylin and Eosin (H-E) at two and four weeks.

Histological section, at × 100 magnification, of control (A), ALN (B), LIPUS (C), and ALN+LIPUS (D) at two weeks of treatment. The osteotomy line was observed as a disruption of the growth plate (arrowhead). Rich, mature trabecular bone bonding (arrows) was observed in the ALN (F) and ALN+LIPUS (H) groups compared with the Control (E)or LIPUS (G) groups.

At two weeks of treatment, ALN and combined treatment with ALN and LIPUS show some trabecular bone at the osteotomy site (arrows). At four weeks of treatment, ALN alone and the combined treatment reveal much trabecular bone (arrows) at the osteotomy site, and we have identified that a cancellous bone union is completed.

	Control	ALN	LIPUS	ALN+LIPUS		Two factor factorial ANOVA		
						ALN	LIPUS	Interaction
Bone union rate (%)								
2 wks	28.99 ± 10.06	38.86 ± 12.29	30.81 ± 14.88	42.46 ± 22.53		p = 0.060	p = 0.68	N.S.
4 wks	23.95 ± 12.30	28.80 ± 11.71	22.10 ± 10.25	44.52 ± 10.20$^{\alpha,\beta}$		p = 0.023	p = 0.20	N.S.
All values are mean ± SD α, p = 0.048 vs. control group; β, p = 0.029 vs. LIPUS group by one-way ANOVAwiththe Scheffe post hoc test								

Table 3: Bone union rate at the osteotomy site of proximal tibia.

Two treatments with ALN and/or LIPUS did not affect the cancellous bone union. ALN treatment for four weeks significantly increased (p = 0.023, two factor factorial ANOVA) cancellous bone union. The cancellous bone union of the ALN+LIPUS group at four weeks was significantly higher than the control (p = 0.048) or LIPUS (p = 0.029)groups, according to the Scheffe multiple comparison test.

Biomechanical analysis of the right distal femur (Table 4):

	Control	ALN	LIPUS	ALN+LIPUS		Two factor factorial ANOVA		
						ALN	LIPUS	Interaction
2 wks								
ultimate load (N)	143.6 ± 44.9	159.4 ± 34.0	165.9 ± 42.8	221.8 ± 30.5¶		p = 0.029	p = 0.057	N.S.
stiffness (N/mm),	174.4 ± 38.1	191.9 ± 77.2	149.2 ± 27.0	4204.5 ± 50.2		p =0.15	p = 0.62	N.S.
breaking energy (Nm)	355.1 ± 105.9	410.4 ± 50.8	360.2 ± 43.1	496.5 ± 39.5§*		p = 0.010	p = 0.18	N.S.
4 wks								
ultimate load (N)	201.0 ± 46.2	190.9 ± 14.1	185.6 ± 40.6	202.3 ± 36.6		p = 0.85	p = 0.88	N.S.
stiffness (N/mm),	235.4 ± 52.2	226.3 ± 24.2	240.6 ± 25.8	259.4 ± 26.7		p = 0.76	p = 0.17	N.S.
breaking energy (Nm)	429.5 ± 70.9	422.9 ± 65.8	417.1 ± 50.3	453.7 ± 26.1		p = 0.59	p = 0.71	N.S.
All values are mean ± SD ¶, p = 0.048 vs. control; §, p = 0.019 vs. control; and*, p = 0.024 vs. LIPUS by one-way ANOVA with the Scheffepost hoc test								

Table 4: Mechanical properties of cancellous bone.

ALN treatment for two weeks significantly increased ultimate load (p = 0.029) and breaking energy (p = 0.010). The ALN+LIPUS group showed a significant increase in ultimate load compared with the control group (p = 0.048), and in breaking energy compared with the control and LIPUS groups (p = 0.019 and p = 0.024, respectively) at two weeks. At four weeks, ALN and/or LIPUS treatment did not affect the mechanical properties of the distal femur.

Discussion

This study investigated the effects of ALN and/or LIPUS on cancellous bone and its healing at proximal tibial osteotomy sites in OVX-rats. ALN increased BMD and BV/TV while decreasing Oc. N/BS after two and four weeks of treatment. LIPUS also increased BV/TV, but not BMD, with a decrease in Oc.N/BS observed at two weeks of treatment. Combined treatment with ALN and LIPUS significantly increased BV/TV more than LIPUS treatment alone at two weeks. However, the cancellous bone union rate was not improved with the combined treatment at two weeks. At four weeks of treatment, neither ALN nor LIPUS treatment improved cancellous bone union; however, combined treatment with ALN and LIPUS increased cancellous bone union at the proximal tibial osteotomy site in OVX rats.

The present study demonstrated that ALN did not inhibit cancellous bone healing at the proximal tibial osteotomy site in an osteoporotic animal model. A recent animal study also showed that ALN increased the ultimate force required for screw insertion into the tibial metaphysis in mice [31]. These animal studies demonstrated that ALN did not impair healing or remodelling of damaged cancellous bone. In clinical studies of BP effects on cancellous

bone injuries, zoledronic acid did not cause a significant increase in the incidence of delayed healing or the non-union rate in low-trauma hip fractures compared with placebo [32]. BP use did not significantly affect the clinical results during conservative treatment for osteoporotic spinal fractures [33]. However, the occurrence of an intravertebral cleft sign was associated witha history of BP use [33]. Although these results indicate that BPs do not impair cancellous bone healing or union in osteoporotic conditions in animals and clinical studies, further study will be needed to confirm the effects of ALN on cancellous bone healing at several different sites in osteoporotic conditions.

Several previous studies have reported the mechanisms of LIPUS in fracture healing. LIPUS exerts positive effects on signal transduction, gene expression, cell differentiation, and extracellular matrix synthesis and mineralization [34-37]. Furthermore, a rodent study has shown that LIPUS produces an anabolic effect during each sequential phase of the healing process: during the initial inflammatory stage, soft callus formation, hard-callus formation, and remodeling [13]. Additionally, recent work has shown that LIPUS may accelerate fracture healing by enhancing callus formation and maturation [38]. These mechanisms contributed to suppress bone resorption, increase bone volume, and improve cancellous bone union following ALN treatment in this study.

Our previous study using aged rats demonstrated that ALN did not impair, and LIPUS improved cancellous bone union at proximal tibial osteotomy sites at two and four weeks post-osteotomy [17]. However, combined treatment with ALN and LIPUS did not affect cancellous bone union in aged rats [17]. On the other hand, LIPUS did not improve cancellous bone healing at two and four weeks of treatment in OVX rats. Furthermore, only combined treatment with ALN and LIPUS for four weeks, but not for two weeks, improved cancellous bone healing in OVX rats. The results of the present and previous studies indicate that restoration of cancellous bone volume isrequired for healing of cancellous bone after osteotomy under osteoporotic conditionsproduced by OVX.

ALN monotherapy and combined treatment with ALN and LIPUS produced significant increases in bone strength inthe distal femoral metaphysis. This result indicates that the ability of ALN to increase bone strength under osteoporotic conditions is not limited to regions of cancellous bone damage. In clinical situations, the prevention of consequent fractures arising from bone fragility isvery important in elderly people. Based on the results of the present study, ALN treatment should be initiated as early as possible after osteoporotic vertebral or femoral fractures to prevent subsequent fractures.

It is known that ALN monotherapy can delay the healing of non-spinal fractures in humans [39]. Nozaka et al. reported that LIPUS with teriparatide therapy may become a useful option in the treatment of elderly patients with lower limb fractures [40]. However, no report has evaluated the clinical effects of ALN and LIPUS on cancellous bone healing in osteoporotic patients. The present study indicated that this combined therapy has potential for use in osteoporotic fractures.

The primary limitation of this study is that osteotomy at the proximal tibia may not correspond to cancellous bone fractures encountered clinically, such as those involving the vertebra, hip, knee, or shoulder. We have utilized this osteotomy procedure to evaluate cancellous bone healing as a model of periarticular fractures.

Conclusion

ALN increased bone volume and did not prohibit cancellous bone repair at the osteotomy of proximal tibial metaphysis in OVX rats. LIPUS also stimulated cancellous bone recovery at the osteotomy site in OVX rats. ALN and LIPUS combination therapy is a good optionfor treating cancellous bone fractures under osteoporotic conditions.

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Conflict of interest

All authors have no conflict of interest to declare.

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