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Original article

Effects of combined therapy of alendronate and low-intensity pulsed ultrasound on metaphyseal bone repair after osteotomy in the proximal tibia of glucocorticoid-induced osteopenia rats

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ABSTRACT

Objectives: Glucocorticoid (GC) treatment inhibits activation of runt-related transcription factor 2 (Runx2), which is essential for osteoblast differentiation from stem cells. As a result, GC treatment results in bone loss, GC-induced osteoporosis (GIO), elevated fracture risk, and delayed bone healing. Bisphosphonates such as alendronate (ALN) are recommended for treating or preventing GIO, and low-intensity pulsed ultrasound (LIPUS) facilitates fracture healing and maturation of regenerated bone. Combined therapy with ALN and LIPUS may stimulate cancellous bone healing in GIO rats. Here, we examined the effect of ALN and LIPUS on cancellous bone osteotomy repair in the proximal tibia of GIO rats.

Methods: Prednisolone (10 mg/kg body weight/day) was administered for 4 weeks to induce GIO in 6-month-old female Sprague-Dawley rats. Tibial osteotomy was then performed and daily subcutaneous injection of ALN (1-μg/kg body weight) was subsequently administered alone or in combination with LIPUS (20 min/day) for 2 or 4 weeks.

Results: ALN significantly increased bone mineral density (BMD) at 2 and 4 weeks, and ALN + LIPUS significantly increased BMD at 4 weeks. Bone union rates were significantly increased after 2 and 4 weeks ALN and ALN + LIPUS treatment. Lastly, ALN and ALN + LIPUS significantly increased the proportion of Runx2 positive cells at 4 weeks.

Conclusions: ALN monotherapy and combined ALN and LUPUS treatment augmented BMD and stimulated cancellous bone repair with increased Runx2 expression at the osteotomy site in GIO rats. However, the combined treatment had no additional effect on cancellous bone healing compared to ALN monotherapy.

1. Introduction

Long-term administration of glucocorticoid (GC) results in significant bone loss, which can be as high as 8%–12% in the first several months of GC therapy and thereafter decreases by 2%–4% per year as long as GC therapy is continued [1]. GC therapy induces a form of osteoporosis known as glucocorticoid-induced osteoporosis (GIO), which is associated with bone loss as well as a higher fracture risk [2–4]. In addition to GIO, GC therapy causes a delay of fracture healing. Waters et al. [5] reported that the bone union rate and callus formation at the ulnar osteotomy site were delayed at 6 weeks after osteotomy in rabbits subjected to 2 months of prednisone treatment. Furthermore, several studies have reported that GC inhibits fracture healing in animal models [6–8]. Thus, it is very important to treat fractures in GIO patients completely and quickly, as these patients fracture easily and show impaired/delayed fracture healing. In addition to the treatment of fracture sites in GIO patients, treatment of general bone fragility in GIO patients is also very important to prevent subsequent insufficiency fractures.

To prevent or treat GIO, bisphosphonates are the first choice of the American College of Rheumatology recommendations [9] and

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the Japanese Society for Bone and Mineral Research [10]. Alendronate (ALN), which is one of the bisphosphonates, is commonly used for GIO prevention or treatment. Saag et al. [11] reported that ALN suppressed the decrease in bone mineral density (BMD) of the lumbar spine, proximal femur and trochanter in GIO patients. Furthermore, Adachi et al. [12] also reported that ALN decreased the incidence of vertebral fractures in GIO patients. However, concern could be raised that ALN treatment may impair healing process in GIO induced fragility fractures, because it exerts its suppressive effects on bone turnover.

Low-intensity pulsed ultrasound (LIPUS) is one of the methods used to accelerate fracture healing in fresh fractures and nonunions [13–15]. Our previous studies have demonstrated that LIPUS facilitates maturation of the regenerated cancellous bone at the osteotomy site of proximal tibia in aged rats [16] and combined treatment with ALN and LIPUS increased cancellous bone repair and bone strength in ovariectomized rats [17]. It has been considered that LIPUS is only useful method to accelerate a local fracture healing in delayed union of patients, who have a worse bone quality such as GIO.

However, to our knowledge, there are no studies investigating the combined effects of ALN and LIPUS on bone repair at the cancellous bone osteotomy of the proximal tibia in GIO rats. The present study aimed to investigate the effects of ALN and/or LIPUS on cancellous bone healing at the osteotomy site of the proximal tibia in GIO rats. We hypothesize that combined therapy with ALN and LIPUS produces a positive effect in stimulating cancellous bone healing in GC treated rats.

2. Methods

2.1. Animals

Six-month-old female Sprague-Dawley rats (Charles River Laboratory Inc., Kanagawa, Japan) were housed in a controlled environment at 22 °C with a 12-h light/dark cycle. The rats were allowed free access to water and pair-fed standard food (CE-2; Clea Japan Inc., Tokyo, Japan) containing 1.14% calcium, 1.06% phosphorus, and 250 IU vitamin D3 per 100 g.

2.2. Experimental design (Fig. 1A)

We administered prednisolone to create the glucocorticoid-induced osteopenia (GIO) rats [18]. Prednisolone (prednisolone sodium succinate, Predonine; Shionogi, Osaka, Japan) was dissolved in saline and injected subcutaneously at a dose of 10-mg/kg body weight (BW)/day 5 times a week for 4 weeks. The rats (n = 66) were then randomized into 4 groups: (1) control group (saline administration with sham-LIPUS, n = 8 and 6 at 2 and 4 weeks, respectively), (2) LIPUS group (saline administration with LIPUS, n = 6 at 2 and 4 weeks), (3) ALN group (ALN administration with sham-LIPUS, n = 7 and 6 at 2 and 4 weeks, respectively), and (4) ALN + LIPUS group (ALN administration with LIPUS, n = 6 and 7 at 2 and 4 weeks, respectively). Fourteen rats were died during the experiment due to the anesthesia for LIPUS treatment. A cancellous bone osteotomy was performed on the right proximal tibia of each rat as previously described [19]. Briefly, a lateral parapatellar incision was made from the knee joint of the right hind limb through the proximal half of the tibia. An incomplete midsagittal osteotomy was performed from the joint surface around one-quarter of the proximal tibia, without extending to the caudal cortex using an electrical power saw (Yoshida Medical Inc., Tokyo, Japan). The site of the osteotomized tibia was closed using a nonabsorbable suture.

Postoperatively, there were no rats with an abnormal gait. ALN administration and/or LIPUS were started from the third day after the osteotomy and continued until the rats were sacrificed at 2 or 4 weeks. The right tibia from each rat was harvested and fixed in 10% neutral buffered formalin. All animal experiments were approved by the "Guidelines for Animal Experiments" of our institute (IACUC number: a-1-2609).

2.3. ALN administration

A solution of ALN (Wako Pure Chemical Co., Ltd, Osaka, Japan) was prepared in saline at a concentration of 0.02 mg/mL. Rats in the ALN and combination groups received a daily subcutaneous injection of ALN (1-μg/kg BW) for 7 days a week. This dose of ALN was equal to the dosage used in humans (5 mg/day) by oral administration, which is an approved dosage in Japan [20] and was chosen to be in accordance with that used in previous animal studies [16,17]. Saline was selected as a vehicle control, and 0.2 mL of saline was injected subcutaneously in the control and LIPUS groups. BWs were measured weekly and the injection dosages were adjusted accordingly.

2.4. Ultrasound intervention (Fig. 1B)

LIPUS was provided by a Sonic Accelerated Fracture Healing System (SAFHS; Teijin Pharma, Tokyo, Japan). LIPUS signal strength and duration of treatment were consistent with the recommended clinical conditions for this device. The ultrasound signal generated with a transducer consisted of a burst width of 200 μs containing 1.5 MHz sine waves at a frequency of 1.0 kHz, and a spatial average-temporal average intensity of 30 mW/cm^2. Rats were anesthetized with an intraperitoneal injection of ketamine (20-mg/kg BW) (Sankyo, Tokyo, Japan) and xylazine (1.5-mg/kg BW) (ZENOAQ, Fukushima, Japan) before exposure to LIPUS or sham-LIPUS 20 min per day for 7 days a week. Sufficient gel was used during the application of the ultrasound, and a rubber band was employed to fix the transducer against the antero-medial side of the osteotomized tibia such that the LIPUS could be routinely and consistently applied at the healing site.

2.5. Measurement of BMD

BMD of the entire excised tibia was measured by dual-energy X-ray absorptiometry (DXA, Hologic QDR-4500; Hologic, Marlborough, MA, USA) in the anterior plane. Bones were scanned in the "small animal" scan mode, with the "regional high-resolution" scan option. The region of interest (ROI) was 20 mm in length from the proximal edge of the tibia and the total width of the tibia [16,17].

2.6. Sample preparation

After BMD measurements, the right proximal half of the tibia from each rat was decalcified with neutral 10% ethylene diaminetetraacetic acid for approximately 4 weeks and embedded in paraffin. Three micrometer-thick mid-frontal slices were then sectioned and stained with Hematoxylin and Eosin (H&E) for cancellous bone histomorphometry.

2.7. Bone histomorphometry

Bone histomorphometric analysis at the proximal tibia including osteotomy site with a magnification of × 200 was performed with a semiautomatic graphic system (Histometry RT CAMERA; System Supply, Nagano, Japan). Measurements were obtained at 400 μm caudally from the lowest point of the growth plate and 100 μm medially from the endosteal surface (Fig. 1C). The histomorphometric cancellous bone per tissue volume (BV/TV; %),