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ORIGINAL ARTICLE

Optimal administration frequency and dose of teriparatide for acceleration of biomechanical healing of long-bone fracture in a mouse model

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Abstract

Despite preclinical studies demonstrating the effectiveness of teriparatide for skeletal repair in small animals, inconclusive data from clinical trials have raised questions regarding the optimal teriparatide dosing regimen for bone repair. To address this, we assessed the effect of teriparatide frequency and dose on long-bone healing using a mouse femur osteotomy/fracture model. Eight-week-old male ICR mice were subjected to open femur osteotomies, then randomized into following five groups ($n = 8$ per group): vehicle; low dose/high frequency: 3 μg/kg/dose, 3 times/day; low dose/low frequency: 9 μg/kg/dose, 1 time/day; high dose/high frequency: 9 μg/kg/dose, 3 times/day; high dose/low frequency: 27 μg/kg/dose, 1 time/day. Skeletal repair was assessed by microcomputed tomography, mechanical testing, and histology 4 weeks after surgery. High-dose and/or high-frequency teriparatide treatment increased callus bone volume but failed to have a significant impact on the biomechanical recovery of fractured femurs, possibly because of impaired cortical shell formation in fracture calluses. Meanwhile, low-dose/low-frequency teriparatide therapy enhanced callus bone formation without interfering with cortical shell formation despite a lesser increase in callus bone volume, leading to significant two and fourfold increases in ultimate load and stiffness, respectively. Our findings demonstrate that administering teriparatide at higher doses and/or higher frequencies raises fracture callus volume but does not always accelerate the biomechanical recovery of fractured bone, which points to the importance of finding the optimal teriparatide dosing regimen for accelerating skeletal repair.

Keywords Teriparatide · Fracture · Dose · Frequency · Parathyroid hormone (1-34)

Introduction

Intermittent administration of teriparatide (TPD), an active recombinant human peptide sequence of the parathyroid hormone, increases bone mass in patients with osteoporosis [1]. Based on this anabolic property, a number of animal studies have demonstrated that TPD also enhances skeletal repair, regardless of the skeletal site and mode of bone healing [2–7]. Moreover, clinical case reports [8–10] and some randomized clinical trials [11, 12] of off-label TPD use for enhancement of bone repair have suggested that the drug could be effective as an adjuvant for fracture repair. However, although a phase 2 clinical trial of distal radial fractures in postmenopausal women has shown that 20 μg/day TPD accelerates the time to radiographic healing from 9.1 to 7.4 weeks versus saline controls, it failed to meet its primary prospective endpoint with 40 μg/day TPD [12]. The efficacy of therapy in that clinical trial raised several questions that warrant further investigation into the TPD dosing regimen for fracture repair. One important question is whether the osteoporosis dosing regimen is optimal for enhancing bone repair. Given that the effect of TPD on bone metabolism differs according to the frequency [13, 14] and dose [15] of administration, the optimal TPD dosing regimen for fracture repair might differ from that of osteoporosis treatment.

In the present study, we tested the efficacy of TPD frequency (1 or 3 times/day) and dose (9 or 27 μg/kg/day) on

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a preclinical mouse femur osteotomy/fracture model to elucidate the optimal dosing regimen for fracture repair. Our results will provide important information for facilitating the clinical application of TPD therapy.

Materials and methods

Animals and osteotomy model

All animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). All animal protocols were approved by the Institutional Committee on Animal Resources. Male ICR mice ($n = 40$, 7 weeks of age; CLEA Japan, Inc., Tokyo, Japan) were maintained at 20 °C on a 12-h light/12-h dark cycle with free access to water and mouse food. Following a 1-week adaptation period, all mice underwent a unilateral osteotomy of the femur. Briefly, transverse osteotomy at the mid-shaft femur was performed using a threaded bone saw (MC1111 Tip tool, SANSYO, Tokyo, Japan), and then the osteotomized femur was stabilized with an intramedullary titanium wire ($\varnothing = 0.60$ mm; TERUMO, Tokyo, Japan). This model was modified from the femur fracture model reported previously [16].

Experimental design

Recombinant human TPD (Forteo®; Lilly Inc., Indianapolis, IN) or phosphate-buffered saline was administered to mice via subcutaneous injections.

Dosing regimens were based on information from earlier animal studies. Most rodent models employ TPD doses of 5–200 (typically 40) μg/kg body weight per day to achieve anabolic effects on skeletal repair, and 20 μg/day (equivalent to 0.25–0.4 μg/kg/day in a 50–80-kg person) for clinical treatment of osteoporosis. Recent reports have demonstrated that mice require subcutaneous TPD injections 2–4 times/day to mimic the human skeletal response to once daily dosing of TPD, and that once daily TPD injections in mice correspond to once weekly TPD regimens in humans with regard to histological response [17]. Accordingly, five experimental groups ($n = 8$ in each group) were given the following regimens:

(1) Control (vehicle): saline injection.
(2) Low dose/high frequency (L/H): 3 μg/kg, 3 times/day (total daily dose: 9 μg/kg).
(3) Low dose/low frequency (L/L): 9 μg/kg, 1 time/day (total daily dose: 9 μg/kg).
(4) High dose/high frequency (H/H): 9 μg/kg, 3 times/day (total daily dose: 27 μg/kg).
(5) High dose/low frequency (H/L): 27 μg/kg, 1 time/day (total daily dose: 27 μg/kg).

Thus, the treatment regimens were defined by daily dose (9 or 27 μg/kg/day) and frequency of administration (1 or 3 times/day). Treatment began 1 day after surgery and lasted 4 weeks. The mice were then killed and bilateral femurs were harvested. The fractured femurs and contralateral intact femurs were subjected to microcomputed tomography (micro-CT) analysis and biomechanical testing.

Micro-CT analysis

Femurs were scanned individually by micro-CT (R_mCT2; Rigaku, Tokyo, Japan) at a 10-μm isotropic resolution. Three-dimensional reconstruction of mineralized tissue and quantitative analysis of fracture calluses were performed using TRI-BONE software (RATOC System Engineering, Tokyo, Japan). The region of interest was the osteotomy site, including the area extending 2.5 mm proximally and distally from the center of the gap. A total of 250 micro-CT axial scans were taken. The total bone volume (TV) was quantified first and then original total bone volume (TVoriginal) was calculated by manually segmenting the original bone from the surrounding mineralized callus. The difference between TV and TVoriginal was computed to determine the callus volume. The threshold for segmentation of the mineralized callus was 200 mg of hydroxyapatite/cm^3, based on a phantom comprising known hydroxyapatite concentrations [18]. The bone mineral content of callus (BMC_{callus}) was measured and the bone mineral density of callus ($BMD_{callus} = BMC_{callus}$/[callus volume]) was calculated [19].

To investigate the effect of various TPD dosing regimens on systemic bone, the contralateral intact femurs were scanned by micro-CT and evaluated in accordance to guidelines described by Bouxsein et al. [20]. A 1000-μm volume of interest (100 slices) encompassing the region of the distal metaphysis, starting from 300 μm proximal to the growth plate, was used to assess trabecular bone morphology and cortical thickness.

Biomechanical testing

A three-point bending breakdown test was performed at the fracture sites using a load mechanical universal testing machine (RTC-1310; AND Corp., Tokyo, Japan). Each femur was placed with its anterior surface facing upward on the two lower support bars 8 mm apart, and the loading bar was positioned at the fracture site (anteroposterior position). The load was applied at a rate of 1 mm/min until breakage. The ultimate load (N) and stiffness (N/mm) were calculated from a load–deformation curve [4, 21].

Histological evaluation

For dynamic bone formation analysis, calcein (20 mg/kg; Dojindo Laboratories, Kumamoto, Japan) was injected subcutaneously 4 and 2 days before the mice were euthanized. After biomechanical testing, the femurs were fixed in 70% ethanol, and stained with Villanueva Bone Stain. These specimens were then subjected to undecalcified tissue processing [22]. The specimens were embedded in methyl methacrylate (Wako Chemicals, Kanagawa, Japan) and sectioned at 5 µm in the axial plane. Two axial planes (500 µm proximal and distal to the osteotomy lines in the femurs) were examined by fluorescence microscopy (BX53; Olympus, Tokyo, Japan) to evaluate the dynamic parameters of bone formation.

Histomorphometric analysis was performed using ImageJ (NIH, Bethesda, MD). The measured parameters for cancellous bone in fracture calluses included total tissue volume (callus volume), bone volume (B.Ar), bone surface (B.Pm), single- and double-labeled surfaces (sL.Pm and dL.Pm, respectively), and interlabeled width. These data were used to calculate the bone formation rate to B.Pm ratio (BFR/B.Pm), mineral apposition rate (MAR), and percent bone volume (B.Ar/callus volume) in accordance with the standard nomenclature proposed by Dempster et al. [23].

To compare bone volume and the degree of completion of cortical shell formation in the fracture calluses among experimental groups, we measured surface porosity of the cortical shells in the fracture calluses.

Statistical analysis

All statistical analyses were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and all values are presented as mean ± SD. Values of $p < 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

Results

The fracture model was successfully established in all 40 mice. All the mice survived surgery and there were no postoperative infections.

Biomechanical differences in the effect of teriparatide frequency and dose on femoral fracture repair

To elucidate the effect of TPD dosing regimens on accelerating murine fracture healing, we injected animals with 9 or 27 µg/kg/day TPD for 4 weeks, at a frequency of 1 or 3 times/day. Because faster restoration of the biomechanical integrity of fractured bone is the primary goal of TPD therapy, we assessed this outcome 4 weeks after surgery. At that point, the biomechanical properties of fractured femurs in the vehicle mice recovered only to approximately 20% that of unfractured femurs (Fig. 1a, b). Low-dose TPD treatment increased ultimate load and stiffness, whereas high-dose TPD administration did not improve biomechanical properties despite the formation of oversized calluses (Fig. 1a, c). Frequency of TPD administration also affected the biomechanical recovery of osteotomized femurs. Femurs from the L/L group had 2.7 times the ultimate stress and 3.8 times the stiffness of those from the vehicle group, whereas femurs from the L/H group had only 1.4 times the ultimate stress and 1.3 times the stiffness of those from the vehicle group.

Radiographic differences in the effect of teriparatide frequency and dose on femoral fracture repair

The effects of TPD on bony callus formation were assessed by micro-CT analysis at 4 weeks post surgery (Fig. 2a).

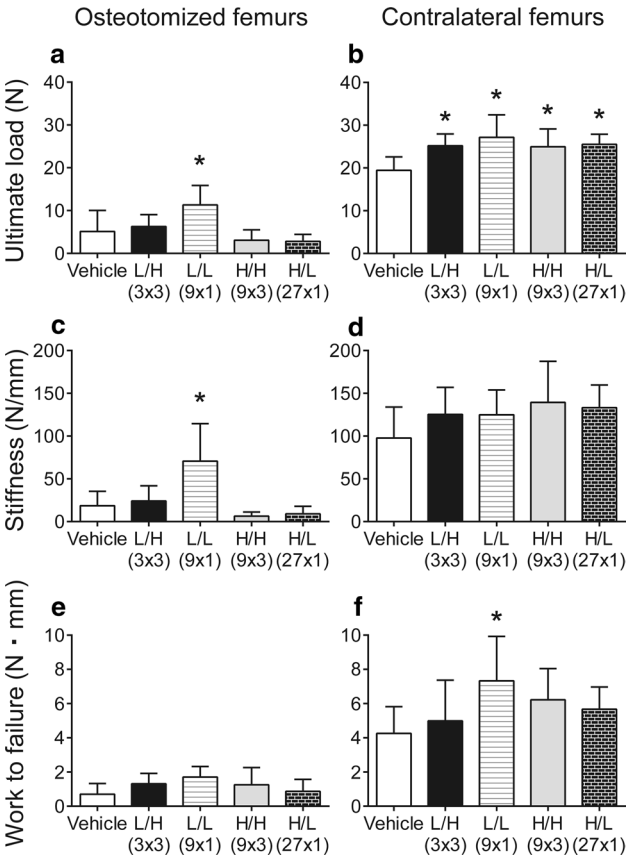

Fig. 1 Biomechanical properties of osteotomized (**a**, **c**, **e**) and contralateral healthy femurs (**b**, **d**, **f**) at 4 weeks post-osteotomy. Ultimate load (**a**, **b**), stiffness (**c**, **d**), and work to failure (**e**, **f**) were quantified by a three-point bending test. Values are mean ± SD ($n = 8$); *$p < 0.05$

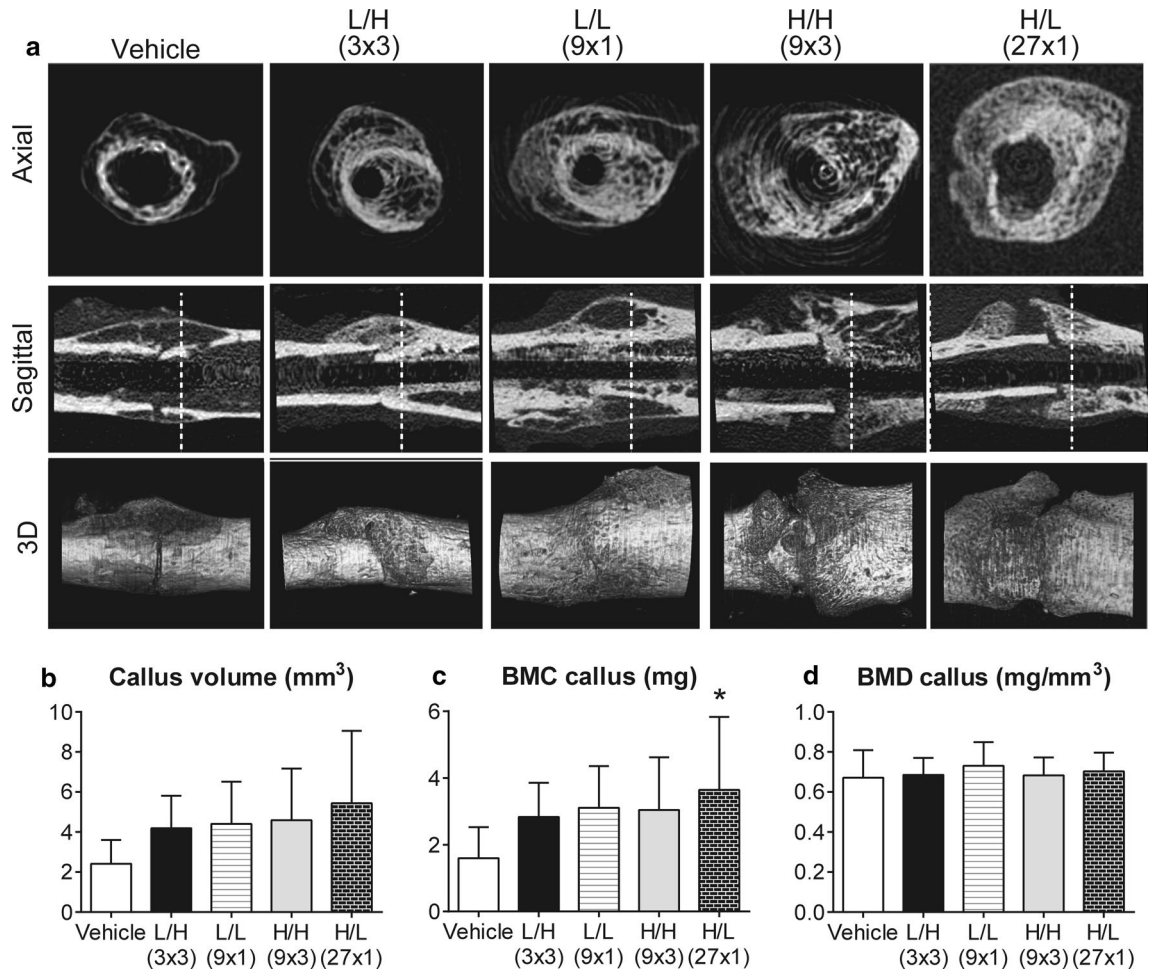

Fig. 2 Radiographic analysis of osteotomized femurs at 4 weeks postsurgery. **a** Representative micro-CT images of the osteotomized region of femurs: axial view, sagittal view, and 3D rendering image for various treatment regimens. Callus volumes (**b**) and bone mineral content (BMC) of calluses (**c**) were quantified, and bone mineral densities (BMDs) of calluses (**d**) were calculated. Values are mean ± SD ($n = 8$); *$p < 0.05$

Saline-treated mice already had calcified calluses at the fracture sites. All the TPD treatment groups had higher callus volumes and BMC_{callus} than the vehicle group, although statistically significant increases were only observed only in the H/L group (Fig. 2b, c). With respect to bone mineral density in fracture calluses, TPD treatment did not have significant effect on BMD_{callus} regardless of treatment regimens (Fig. 2d). Most importantly, the fracture lines tended to be more visible on micro-CT photographs in high-dose and/or high-frequency TPD administration groups despite having macroscopically larger fracture calluses than those of the L/L group.

To confirm the effect of TPD therapy on uninjured normal bone, we performed micro-CT bone morphometric analyses on contralateral femurs (Fig. 3a). All TPD treatment groups except the L/H group had higher bone volume and improved trabecular bone microstructure compared to the vehicle group. Regarding cortical bone, TPD therapies tended to increase cortical thickness (Ct.Th) but only the H/L group showed significant increases in Ct.Th compared to the vehicle group. It should be noted that we observed increased cortical porosity in the axial images of femoral diaphyses from the H/H group (Fig. 3b–d).

Histological differences in the effect of teriparatide frequency and dose on femoral fracture repair

To better understand the mechanism responsible for differences in biomechanical recovery that are due to TPD frequency and dose, we performed a histological analysis at 4 weeks post surgery (Fig. 4a–s). Consistent with micro-CT data, TPD treatment increased the size of fracture calluses regardless of dose and frequency. Normally, during fracture healing, cartilage that forms at fracture sites is replaced with newly formed bone. In our study, at 4 weeks after fracture, cartilage was already replaced

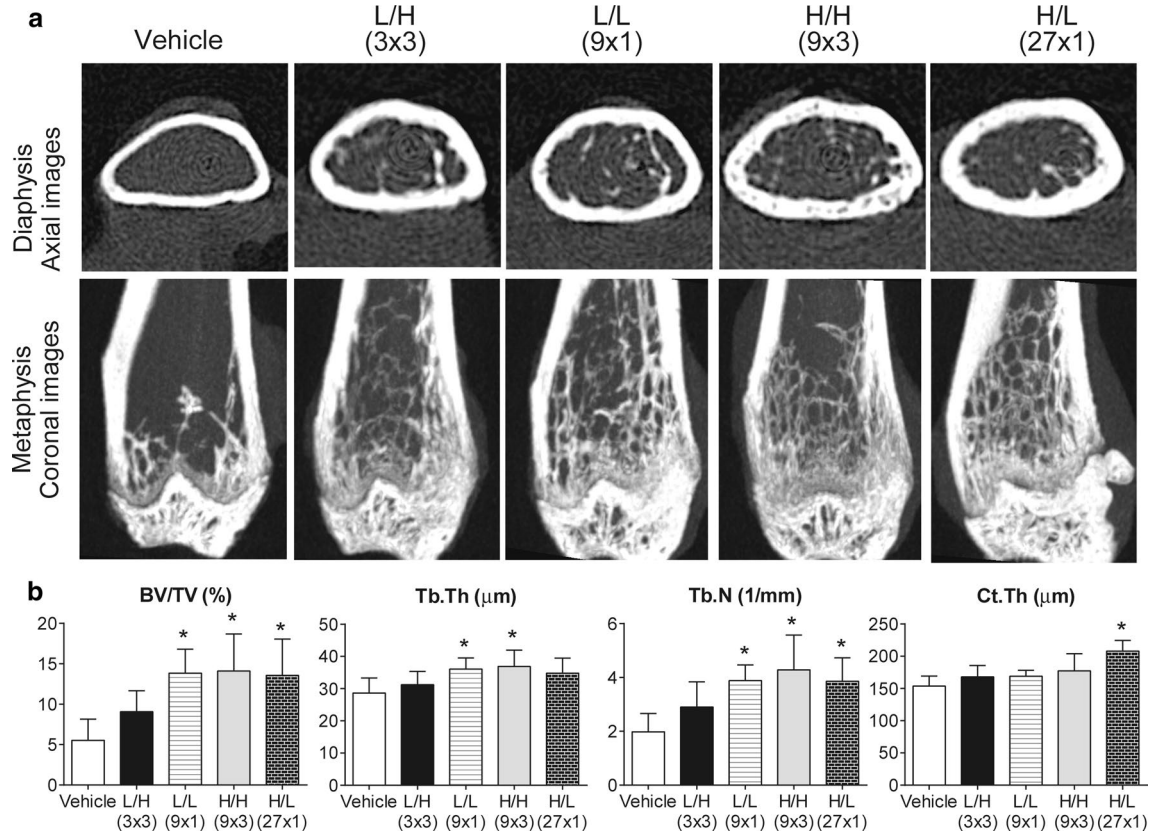

Fig. 3 Radiographic analysis of contralateral unfractured healthy femurs at 4 weeks post surgery. **a** Representative micro-CT diaphysis axial images of femurs and distal metaphysis coronal images for various treatment regimens. **b** The quantitative micro-CT morphometric data of trabecular and cortical bones in distal femurs. Trabecular bone parameters: trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N). Cortical bone parameters include cortical thickness (Ct.Th). Values are mean ± SD ($n = 8$); * $p < 0.05$

with bone in the vehicle group. In all TPD groups except the L/L group, some cartilage tissue remained, albeit in small areas of some specimens. The TPD therapies significantly increased MAR and BFR/B.Pm compared to that of the vehicle group regardless of administration frequency or dose (Fig. 4p, q). Among TPD treatment groups, H/H group had higher BFR/B.Pm ratios compared to that of the L/L and H/L groups. There were apparent differences in the structure of bony callus tissues, which were composed of abundant trabecular bone in the TPD treatment groups but were composed of cortical shell and small amounts of trabecular bone in the vehicle group. The L/L and H/L groups showed significant increases in B.Ar/callus volume ratios compared to that of the vehicle group (Fig. 4r). With respect to differences in the structure of fracture calluses among TPD treatment groups, cortical shells formed around fracture calluses in the L/L group, whereas other TPD treatment groups had impaired cortical shell formation despite abundant trabecular bone formation. The surface porosity of cortical shells tended to increase with the frequency and dose of TPD. Surface porosity for those of the H/L group was significantly higher compared to those of other groups (Fig. 4s).

Discussion

The effect of TPD therapy on cortical shell formation during fracture healing is of great interest because the process is indispensable, especially for weight-bearing long bones to regain biomechanical integrity sufficient enough to bear the loads applied during daily activity. However, little information exists regarding the effect of frequency of TPD administration on cortical bone modeling and remodeling during fracture healing. Several osteoporosis studies of uninjured bones in monkey and rabbit models [13, 14, 24, 25] have reported that once daily dosing of TPD increases cortical porosity in a dose-dependent manner because of the enhancement of intracortical and endocortical bone remodeling, whereas once weekly dosing does not increase cortical porosity. Yamamoto et al. reported that administration of parathyroid hormone (PTH) 4 times/day mice results in loss

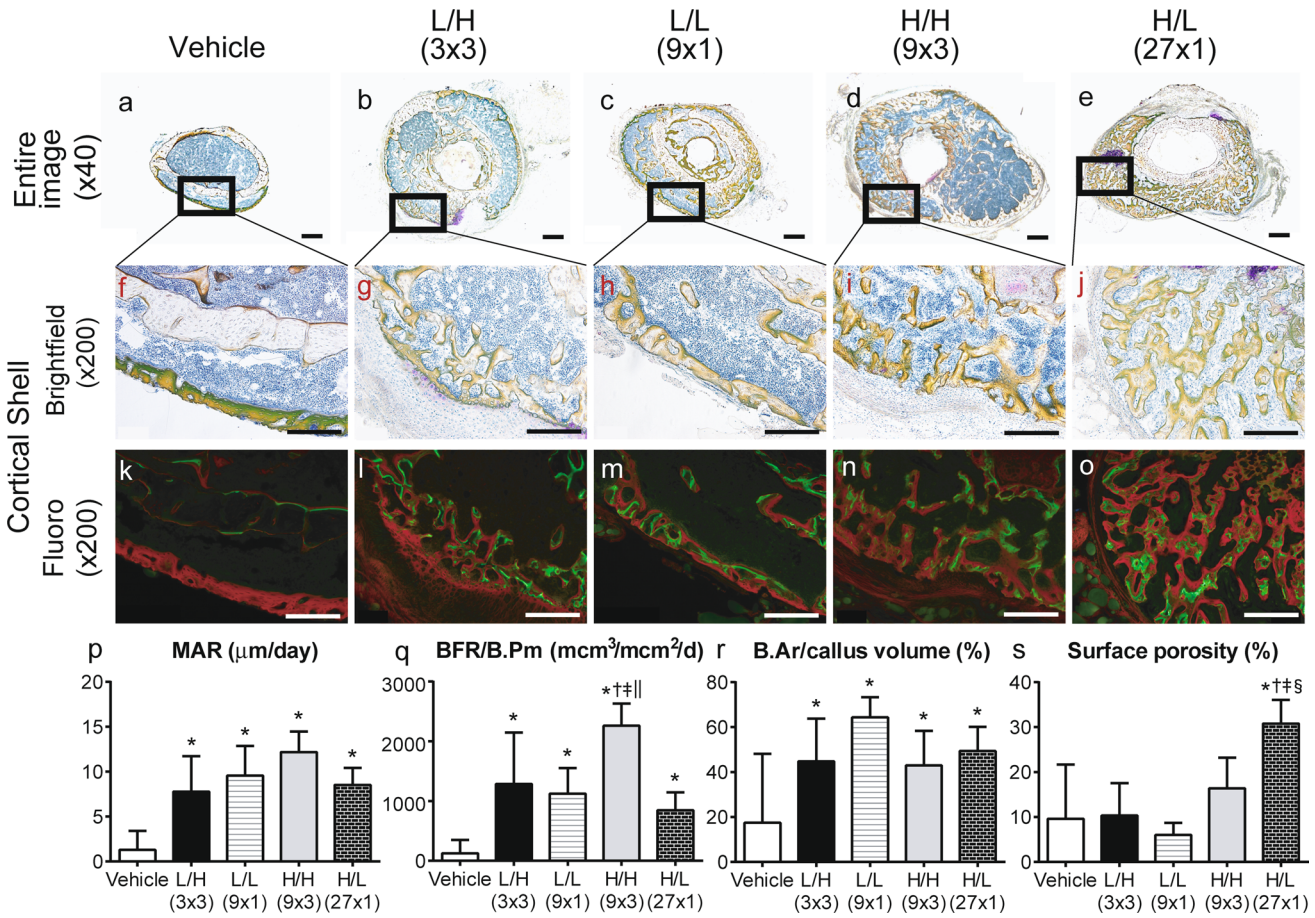

Fig. 4 Histological analysis of fracture calluses at 4 weeks postsurgery. **a**–**p** Representative micrographs of axial sections of fracture calluses treated with vehicle (**a**, **f**, **k**), Low-dose/high-frequency (L/H) teriparatide (**b**, **g**, **l**), low-dose/low-frequency (L/L) teriparatide (**c**, **h**, **m**), high-dose/high-frequency (H/H) teriparatide (**d**, **i**, **n**), and high-dose/low-frequency (H/L) teriparatide (**e**, **j**, **o**). **a**–**e** Low-magnification bright-field images of entire calluses. **f**–**j** and **k**–**o** High-magnification bright-field images and epifluorescent light images of the cortical shell region of fracture calluses, respectively. **p**–**r** Histomorphometry of fracture callus regions. MAR, mineral apposition rate; BFR/B.Pm, bone formation rate per bone surface; and B.Ar/callus volume, bone volume per callus volume. **s** Surface porosity of the cortical shells of fracture calluses. Values are mean ± SD ($n = 6$). *, †, ‡, §, and ‖ indicate a statistically significant difference compared to vehicle, L/H, L/L, H/L, and H/H, respectively. Scale bars: 500 μm (**a**–**e**) and 200 μm (**f**–**o**)

of cortical bone density and increased porosity [17]. In our study, an increase in cortical porosity was observed in contralateral unfractured femurs from the H/H treatment group but not in low-dose and/or low-frequency groups. Together with previous studies, our data indicate that high-frequency TPD therapy works against cortical shell formation during the fracture healing process.

Indeed, frequency of administration had a profound facilitatory effect on the biomechanical recovery of fractured bone. High-frequency TPD therapy enhanced trabecular bone formation and remodeling of original bone but did not improve cortical shell formation at the fracture callus, resulting in reduced enhancement of biomechanical recovery compared to low-frequency TPD therapy. Meanwhile, low-frequency TPD therapy enhanced callus bone formation without interfering with cortical shell formation despite a lower increase in callus bone volume. This suggests that although the oversized fracture calluses induced by high-frequency TPD therapy may appear promising, they do not always contribute to biomechanical recovery in the early phase of fracture healing. Similarly, high-dose TPD increased fracture callus volume but did not enhance the biomechanical recovery of fractured bone. One possible explanation is that higher doses of TPD preferentially enhance chondrogenesis over osteogenesis, leading to oversized calluses with an abundance of cartilage that takes time to be replaced with bone [26].

It is inconclusive which dosing regimen in mice corresponds to the clinical regimen (once daily dosing) in humans. A recent study reported that once weekly injections of 10 and 20 μg TPD are equally effective at promoting bone fracture healing in rats, but the corresponding

human dosing regimen remains unknown [27]. While the half-life of TPD following subcutaneous injection is 60–75 min in humans [28, 29], it is 26–30 min in rats [30]. That difference in metabolism may result in discrepancies in effective dose and/or dosing intervals between humans and rodents. Therefore, data obtained from rodent studies using once daily TPD therapy may lead to misinterpretation of the efficacy of TPD therapy for fracture healing. Indeed, data from earlier studies suggest that mice require more than once daily dosing to produce bone metabolism similar to that seen in humans treated once daily. Dobnig et al. found that given the rapid clearance of TPD in rodents, subcutaneous TPD injections must be given 3 times/day for optimal skeletal response [31]. Takakura et al. estimated that once daily administration in rats is equivalent to once weekly administration in postmenopausal women; and based on areas under the concentration–time curves, a single dose of 6 μg/kg in rats corresponds to a dose two times higher than the 56.5 μg once weekly regimen used in clinical settings in Japan [32]. Thus, from the results of this study, we believe that a lower frequency (e.g., 1 time per several days or 1 time per week) may lead to earlier biomechanical recovery of fractures in clinical settings.

One limitation of this study is that the analyses were performed at only one time point. It would have been better to collect data over multiple time points to confirm the conclusion of this study. However, osteotomized femurs can achieve bone union postoperatively by 9 weeks, even in control mice, while only soft calluses are formed postoperatively at 2 weeks in the mice model we used in this study. The reason we chose the 4-week postoperative time point is that incomplete bony calluses were formed with biomechanical recovery of 20% that of unfractured femurs at that time point. Therefore, we believed that it was the optimal time point for evaluating the status of fracture healing, as well as elucidating the differences in biomechanical recovery and histological healing of osteotomized femurs among a variety of TPD dosing regimens compared to that of the vehicle group. The other limitation is that our femur osteotomy/fracture model used growing mice, whereas previous clinical reports on the effect of TPD on fracture healing were on postmenopausal women with radius fractures. Although we used a highly reproducible rodent model, future studies might require the use of a previously described cancellous proximal tibia osteotomy model to evaluate the effect of TPD on bone healing of both cancellous and cortical bones [33].

In summary, our findings showed that administering TPD at a higher dose and/or higher frequency increases fracture callus volume but does not accelerate the biomechanical recovery of fractured bone. On the contrary, administering TPD at a lower dose and/or lower frequency improves the biomechanical recovery of fractured bone by enhancing fracture callus formation without interfering with cortical shell formation despite a lower increase in callus bone volume.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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