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Author(s)	Shimizu, Tomohiro; Takahata, Masahiko; Kameda, Yusuke et al.
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Vitamin K-Dependent Carboxylation of Osteocalcin Affects the Efficacy of Teriparatide (PTH_{1-34}) for Skeletal Repair

Tomohiro Shimizu[1], *Masahiko Takahata[1], Yusuke Kameda[1], Hiroki Hamano[1], Teppei Ito[2], Hiromi Kimura-Suda[2], Masahiro Todoh[3], Shigeru Tadano[3], Norimasa Iwasaki[1]

[1]Hokkaido University, Department of Orthopedic Surgery, School of Medicine, Sapporo, Japan

[2]Chitose Institute of Science of Technology, Chitose, Japan

[3]Division of Human Mechanical Systems and Design, Faculty of Engineering, Hokkaido University, Sapporo, Japan

[*]To whom correspondence should be addressed:

Masahiko Takahata

Hokkaido University, Department of Orthopedic Surgery, Graduate School of Medicine

Kita-15 Nishi-7, Kita-ku, Sapporo, 060-8638, JAPAN.

Phone: +81-11-716-1161 ext. 5936, Fax +81-11-706-6054

E-mail: takamasa@med.hokudai.ac.jp

Running head: Effect of Teriparatide on Fracture in Vitamin K insufficiency

3 Supplemental figures and 2 Supplemental tables are included with the submission

Disclosure

All authors state that they have no conflict of interest.

Abstract (296/300 words):

Teriparatide (PTH_{1-34}) promotes skeletal repair and increases bone mass. Vitamin K is implicated in bone mineralization as a coenzyme of γ-carboxylase for the Gla proteins, and therefore vitamin K insufficiency caused by malnutrition or therapeutic intake of the vitamin K antagonist warfarin could affect the efficacy of PTH_{1-34} therapy for bone repair. In the present study, we investigated whether vitamin K influences the efficacy of PTH_{1-34} therapy for bone repair in a rat osteotomy model. Female 12-week-old Sprague-Dawley rats were subjected to a closed midshaft osteotomy of the femur and randomized into four groups (n=10 per group): Vehicle, PTH_{1-34} (daily 30 μg/kg/day subcutaneous injection) + solvent (orally, three times a week), PTH_{1-34} + warfarin (0.4 mg/kg/day orally, three times a week), and PTH_{1-34} + vitamin K_2 (menatetrenone, 30 mg/kg/day orally, three times a week). Serum γ-carboxylated and uncarboxylated osteocalcin (Gla-OC and Glu-OC) levels and radiographic healing were monitored every 2 weeks. Skeletal repair was assessed by micro-computed tomography, mechanical testing, and histology at 8 weeks after the surgery. PTH_{1-34} amplified the osteotomy-induced increase in Gla-OC and improved the mechanical properties as well as the volumetric bone mineral tissue density of the fracture callus. Concurrent use of warfarin decreased the response to PTH_{1-34} therapy in terms of mechanical recovery, probably by impairing mineralization due to the lack of Gla-OC. Although the effects of combination therapy with PTH_{1-34} and vitamin K_2 on bone repair did not significantly exceed those of PTH_{1-34} monotherapy in rats fed sufficient dietary vitamin K, postoperative Gla-OC levels were correlated with the mechanical properties of the osteotomized femur in PTH_{1-34} treated rats regardless of the use of warfarin or vitamin K_2. These findings suggest the importance of vitamin K dependent γ-carboxylation of OC for PTH_{1-34} to fully exert its effect on skeletal repair.

Keywords: Vitamin K; Teriparatide; Fracture; Gla-osteocalcin; Mineralization; Parathyroid hormone (1-34)

Introduction

Intermittent administration of teriparatide (PTH_{1-34}), 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 PTH_{1-34} also enhances skeletal repair, regardless of the skeletal site and mode of bone healing [2-6] [7]. Although the effects of this drug on fracture healing are remarkable in normal, skeletally mature animals, whether PTH_{1-34} exerts beneficial skeletal repair effects in pathologic conditions is unknown.

Vitamin K insufficiency might affect the efficacy of PTH_{1-34} therapy for bone repair. Vitamin K is well known to be important for the normal functioning of blood coagulation factors, but it is also thought to be involved in bone metabolism by regulating the activation of bone matrix proteins [8]. Vitamin K acts as a coenzyme of γ-carboxylase, which converts the glutamic acid (Glu) residue in Gla protein to γ-carboxyglutamic acid (Gla) [9-13]. In bone, vitamin K acts on osteocalcin (OC), a highly abundant noncollagenous protein in bone, and Gla-OC is thought to be associated with bone mineralization due to its specific interaction with hydroxyapatite [14].

Mineralization of soft fracture calluses is a critical step for fractured bone to regain mechanical strength and stiffness. Because the hallmarks of PTH_{1-34} therapy during fracture healing are enhanced mineralization and increased callus formation, impaired mineralization may attenuate the efficacy of PTH_{1-34} on bone healing [15]. Given that vitamin K insufficiency is frequently caused by low dietary vitamin K intake and the use of the vitamin K antagonist warfarin as anticoagulant therapy, it is of great interest whether vitamin K insufficiency affects the clinical efficacy of PTH_{1-34} on skeletal repair. In contrast, concomitant use of vitamin K might enhance the efficacy of PTH_{1-34} therapy on skeletal repair based on a recent study showing that vitamin K

promotes bone healing in a rat fracture model [16].

In the present study, we used a rat femoral osteotomy model to investigate whether vitamin K insufficiency or administration of vitamin K affects the efficacy of PTH_{1-34} therapy on bone repair. This study provides important information that facilitates clinical translation of PTH_{1-34} therapy for bone repair.

Materials and methods

Animals and osteotomy model

All animal studies were performed in accordance with protocols approved by the Hokkaido University's Committee on Animal Resources. Female Sprague–Dawley rats (n = 40; 10 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 rat food containing 0.98% Ca, 0.80% Pi, and 15.4 mg/kg vitamin K_3 (Labo MR Stock; Nosan Corporation Life-Tech Department, Yokohama, Japan). Following a 2-week adaptation period to the new environment, all rats were stratified according to bodyweight and underwent a unilateral osteotomy of the femur. Briefly, transverse osteotomy at the mid-shaft femur was performed using a threaded wire saw (MDS36-30 T-saw, MANI, Tochigi, Japan), which was inserted percutaneously, and then the osteotomized femur was stabilized with an intramedullary titanium wire (Ø= 1.0 mm; Synthes, Tokyo, Japan). This closed osteotomy procedure does not preserve periosteum but minimizes other soft tissue damage compared to conventional open osteotomy. This model was modified for the femur from a previous report [17].

Experimental design

After osteotomy, all rats were randomized into four groups within each stratum according to the following treatment schedule: vehicle control group (n = 10), PTH_{1-34} group (n = 10), PTH_{1-34} + warfarin group (n = 10), and PTH_{1-34} + vitamin K_2 group (n = 10). Recombinant human PTH_{1-34} (Forteo®; Eli Lilly, Ltd., Kobe, Japan) at a dosage of 30 μg/kg/day or phosphate-buffered saline was administered to the animals by daily subcutaneous injections [15, 18]. Vitamin K_2 (menatetrenone; Eisai Co., Ltd., Tokyo, Japan) was suspended in fatty acid (Miglyol 812; Mitsuba

Trading, Co., Ltd, Tokyo, Japan) at a dose of 30 mg/ml/kg bodyweight and administered by gavage three times a week [16, 19, 20]. To cancel the effect of dietary vitamin K, warfarin was suspended in distilled water at a dose of 0.4 mg/ml/kg body weight and administered by gavage three times a week [21]. Every two weeks after the surgery, bodyweight was monitored, tail vein blood was collected, and micro-computed tomography (micro-CT) of the femur was performed under inhaled sedation with isoflurane (Forene®). At 8 weeks after the osteotomy, the animals were killed and the treated femora were collected. Micro-CT was performed for all femora. Half of the 10 femora were subjected to biomechanical testing and the other half were subjected to histologic examination.

Serum Gla-OC and Glu-OC levels

Sandwich enzyme-linked immunosorbent assays were performed using commercially available kits to determine the serum Gla-OC and Glu-OC levels (Takara Bio Inc., Shiga, Japan). All samples were assayed in duplicate. A standard curve was generated for each protein, and the absolute concentrations were determined from the standard curve.

Biomechanical testing

A three-point bending breakdown test was performed at the fracture site of the femora using a load mechanical universal testing machine (Model 3365, Instron Corp., Norwood, MA, USA). The femur was placed with its anterior surface facing upward on the two lower support bars 15 mm apart, and the loading bar was positioned at the fracture site (anteroposterior position). The load was applied at a rate of 0.2 mm/min until breakage. The ultimate load (N) and stiffness (N/mm) were calculated from the load–deformation curve. As previously described, the load-displacement data were normalized to obtain intrinsic material properties such as ultimate stress and elastic

modulus, which are independent of cross-sectional size and shape [5].

Micro-CT analysis.

Femora were scanned individually by micro-CT (CT, R_mCT2; Rigaku, Tokyo, Japan) at a 20-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) in accordance with the guidelines described in Bouxsein et al [22]. The region of interest was set at the osteotomy site including the region 2.5 mm extending proximally and distally to the center of the gap with a total of 250 CT axial scans. The total bone volume (BV_{total}) was quantified first and then original bone volume ($BV_{original}$) was calculated by manually segmenting the original bone from the surrounding mineralized callus. The difference between BV_{total} and $BV_{original}$ was computed to determine the mineralized callus volume (BV_{callus}). The threshold for segmentation of the mineralized callus was 200 mg of hydroxyapatite/cm^3, based on a phantom comprising known hydroxyapatite concentrations [23]. Bone mineral content of callus (BMC_{callus}) was measured and tissue mineral density of mineralized callus ($mBMD_{callus} = BMC_{callus}/BV_{callus}$) was calculated [24].

Histology and histomorphometry

For dynamic bone formation analysis, calcein (10 mg/kg, Dojindo Laboratories, Kumamoto, Japan) was injected subcutaneously at 7 days and 2 days, respectively, before the rat was killed. The femora were fixed in 70% ethanol, and stained with Villanueva Bone Stain. These specimens were then subjected to undecalcified tissue processing. The specimens were embedded in methyl-methacrylate (Wako Chemicals, Kanagawa, Japan) and sectioned at 5 μm in the sagittal plane. The osteotomy site of the femur was examined by fluorescence microscopy (BX53,

Olympus, Tokyo, Japan) to evaluate the dynamic parameters of bone formation. Histomorphometric analysis was performed using an Image PRO-Plus (Media Cybernetics, Rockville, MD, USA). The measured parameters for callus bone included total tissue volume (TV), bone volume (BV), osteoid volume (OV), bone surface (BS), osteoblast number, osteoblast surface, single and double labeling surfaces (sLS and dLS, respectively), and inter-label width. These data were used to calculate percent bone volume (BV/TV), percent osteoid volume (OV/BV), osteoblast number (N.Ob/BS), osteoblast surface (Ob/BS), mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR)/BS in accordance with the standard nomenclature proposed by Dempster et al [25].

Statistical Analysis

All data are expressed as means and standard error of the mean (SEM). Comparisons of data among the groups were performed using a one-way analysis of variance and Newman-Kuels tests. A significance level of P less than 0.05 was used for all comparisons. Correlations between serum Gla-OC level and mechanical properties were performed using Pearson product-moment correlation coefficient, with level of significance set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) on a Windows computer.

Results

Gla- and Glu-OC levels during bone repair

Serum levels of Gla-OC and Glu-OC were measured at the time of surgery, and 2, 4, 6, and 8 weeks after osteotomy, and the time course of change in Gla- and Glu-OC levels was assessed in each individual (Fig. 1). The Gla- and Glu-OC data in the vehicle group showed a natural course of changes in Gla- and Glu-OC after bone injury. In the vehicle group, Gla-OC increased by 18% from the baseline at 2 weeks after osteotomy and then decreased from 2 to 8 weeks after osteotomy. PTH_{1-34} treatment amplified the postoperative increase in Gla-OC production. The rats in the PTH_{1-34} group showed 100% to 140% increase in Gla-OC from the baseline at 2, 4, and 6 weeks after surgery. While supplementation of vitamin K_2 did not further change the postoperative course of the Gla-OC levels of PTH_{1-34}-treated rats, warfarin blocked the increasing effect of PTH_{1-34} on Gla-OC and decreased Gla-OC levels to baseline (-50 ± 12 and -88 ± 14% from baseline at 2 and 6 weeks after osteotomy, respectively).

Glu-OC increased during first 4 weeks after the surgery and then decreased from 4 to 8 weeks after the surgery in the vehicle treated rats. While PTH_{1-34} and PTH_{1-34} + vitamin K_2 groups showed no significant effect on the time course of change in Glu-OC, administration of warfarin remarkably upregulated Glu-OC at any time point after the surgery (mean change of Glu-OC at 2 weeks after osteotomy from the baseline; 2238 ± 789% in PTH_{1-34} + warfarin group vs. 185 ± 47% in PTH_{1-34} group).

Effect of vitamin K insufficiency or supplementation on the efficacy of PTH_{1-34} therapy in biomechanical parameters of bone repair

We assessed biomechanical properties of osteotomized femurs at 8 weeks after osteotomy to

allow for adequate healing for the three-point bending test. Consistent with previous reports, PTH_{1-34} administration increased the ultimate stress and stiffness of the osteotomized femur to 2.4 and 1.7 times those in the vehicle group [18, 26]. Concomitant use of warfarin attenuated the effect of PTH_{1-34} administration on the biomechanical properties (ultimate stress and stiffness to 0.5 and 0.6 times those in the PTH_{1-34} group). Meanwhile, concurrent use of vitamin K_2 and PTH_{1-34} showed some additive, albeit statistically insignificant, effects on the biomechanical properties (ultimate stress and stiffness to 1.5 and 1.3 times those in the PTH_{1-34} group). The effects of warfarin and vitamin K_2 on intrinsic material properties of the osteotomized femur in PTH_{1-34}-treated rats, including elastic modulus and bending strength, were similar to those on ultimate stress and stiffness (Table 1). Although we failed to detect a statistically significant difference among the PTH_{1-34} administration groups, we found a statistically significant correlation between Gla-OC levels at 2, 4, and 6 weeks after surgery and the mechanical properties (Table 2).

Effect of vitamin K insufficiency or supplementation on the efficacy of PTH_{1-34} therapy in radiographic parameters of bone repair

To assess the bone repair process after osteotomy, longitudinal micro-CT analysis was performed at 2, 4, 6, and 8 weeks after osteotomy. The effect of PTH_{1-34} on bone repair was apparent on micro-CT reconstruction images by the large and solid callus formation (Fig. 2A) [5, 15, 27]. The gap at the osteotomy site was filled with calcified bone within 6 weeks after osteotomy in PTH_{1-34} and PTH_{1-34} + vitamin K_2 groups, whereas mineralization of the osteotomy gap tended to be delayed in PTH_{1-34} + warfarin group.

To more carefully assess the effect of PTH_{1-34} and vitamin K level on radiographic healing, quantitative analysis of the calcified callus was performed after removal of the intramedullary

metal pin 8 weeks after osteotomy. BV_{callus} and $mBMD_{callus}$ were higher in the PTH_{1-34} administration groups than in the vehicle groups (Fig. 2B, C). Although PTH_{1-34} + warfarin group tended to show lower BV_{callus} and $mBMD_{callus}$ than the other two PTH_{1-34} groups, we failed to detect a significant difference in BV_{callus} and $mBMD_{callus}$ among the PTH_{1-34} administration groups.

Effect of vitamin K insufficiency or supplementation for the efficacy of PTH_{1-34} therapy on histologic parameters of bone repair

To better understand the mechanism responsible for the drug effects on biomechanical properties, we analyzed the histology at 8 weeks after surgery. Similar to the findings of the micro-CT study, the calluses were densely formed (Fig. 3A-D) and BV/TV was greater in the PTH_{1-34} administered groups than in the vehicle group (Fig. 3M). The osteoid, which is unmineralized bone matrix stained red-purple with Villanueva staining, was abundant in the specimens of the PTH_{1-34} + warfarin group (Fig. 3G and K), and the OV/BV was higher in the PTH_{1-34} + warfarin group compared to the vehicle and PTH_{1-34} + vitamin K groups (Fig. 3N). N.Ob/BS, Ob.S/BS, and MS/BS were greater in the PTH_{1-34} administered groups than in the vehicle group but these are similar among PTH_{1-34}, PTH_{1-34} + warfarin, and PTH_{1-34} + vitamin K_2 groups (Fig. 3O and P). Analysis of the BFR revealed that PTH_{1-34} enhanced the mineralization of the fracture callus based on an increase in MAR and BFR/BS (Fig. 3I-L and Q-S) [6] While concomitant use of vitamin K_2 did not further increase MAR and BFR/BS, concomitant use of warfarin blunted the enhancing effects of PTH_{1-34} on MAR and BFR/BS.

Discussion

The present study addressed whether vitamin K levels affect the efficacy of PTH_{1-34} therapy for

bone repair. Although we could not provide direct evidence for the correlation between vitamin K levels and the efficacy of PTH_{1-34} therapy for bone repair, our data suggest that impaired γ-carboxylation of OC, which is induced by vitamin K insufficiency, attenuates the enhancing effect of PTH_{1-34} therapy for biomechanical recovery of osteotomized bone. There are, however, conflicting results with respect to the effect of impaired carboxylation of OC on bone repair. Einhorn et al. demonstrated that despite a significant decrease in γ-carboxylated OC, vitamin K deficiency due to a diet lacking vitamin K did not impair fracture-healing in a rat femoral fracture model [28]. On the other hand, some studies have demonstrated that warfarin, a vitamin K antagonist, retards fracture healing [29]. Given that PTH_{1-34} therapy upregulated Gla-OC level 5-fold greater than vehicle control after the osteotomy, PTH_{1-34}-treated subjects may be more susceptible to vitamin K insufficiency in terms of bone repair.

We also examined whether vitamin K_2 supplementation enhances the efficacy of PTH_{1-34} therapy for bone repair. Although vitamin K_2 therapy promotes long bone repair in rats [16], we demonstrated only a trend for additive effects of PTH_{1-34} and vitamin K_2 on bone repair. A possible reason for this discrepancy is that all of the animals used in this study were fed a normal diet containing vitamin K_3 and were thus thought to have adequate vitamin K levels, suggesting that this combination therapy is not effective in patients taking sufficient dietary vitamin K. Vitamin K_2 may be worth administering in combination with PTH_{1-34} in elderly patients, however, because elderly people are likely to have lower vitamin K levels [30]. Given that vitamin K_2 promotes the differentiation of osteoblasts through SXR receptors, vitamin K_2 might enhance bone healing through a mechanism other than γ-carboxylation of OC [31, 32].

An interesting finding of the present study is that warfarin remarkably decreased Gla-OC levels, but did not significantly reduce the mineral content of fracture calluses in PTH_{1-34}-treated

rats. This indicates that Gla-OC is not critical for mineralization of the fracture callus. Because of its specific interaction with hydroxyapatite, Gla-OC is thought to affect mineralization of the bone matrix [33, 34]. Recent studies assessing the role of OC, however, indicate that OC is not related to mineral deposition but does participate in the growth and maturation of hydroxyapatite. Amizuka et al. demonstrated that warfarin administration did not change histochemical and histomorphometrical appearance of bone but resulted in crystalline particles being dispersed throughout the osteoid without forming mineralized nodules, which could be observed by electron microscopy, in a rat model [35]. Genetic studies showed that OC depletion increases bone mass but do not change the mineral content of bone matrix histologically [36]. In a more sensitive assay of mineralization, Fourier transform infrared spectroscopy analysis revealed that hydroxyapatite crystals in OC-deficient mice were smaller and less perfect compared to wild-type animals, suggesting that OC plays an important role in bone mineral maturation. Our finding that Gla-OC correlated with the mechanical properties of the operative femur indicates that Gla-OC contributes to the biomechanical recovery of fractured bone by regulating the crystallization behavior of hydroxyapatite, such as orientation and crystallinity, which are considered important bone qualitative factors for the mechanical properties of bone.

It should be noted that the dosage of PTH_{1-34} used in this study corresponds to tens or hundreds of multiples of the FDA approved doses of 20 μg/day for treatment of osteoporosis. We selected the dosage of 30 μg/kg body weight PTH_{1-34} per day because a greater dose of PTH_{1-34} is needed to observe significant effects in rodent fracture models possibly due to the species difference. Animal models typically employ doses of 5–200 (average 40, minimum effective dose is 5) μg/kg body weight per day. Because we hypothesized that the impact of vitamin K insufficiency or supplementation became more significant as production of osteocalcin increased by PTH_{1-34}

1 therapy, we selected a promising dose, 30 μg/kg, for anabolic effects on rat bone repair. However,
2 the effect of combination therapy of lower dose PTH_{1-34} and vitamin K on bone healing should be
3 examined in the future study. If vitamin K could minimize the required dosage of PTH_{1-34} to
4 enhance bone healing, the combination therapy would be helpful for clinical translation of PTH_{1-34}
5 therapy for bone repair by overcoming the problems associated with high dose PTH_{1-34} therapy
6 such as high cost, incidence of hypercacemia and osteosarcoma.

7 There are several limitations to this study. First, we measured OC levels but did not measure
8 serum vitamin K levels because of technical difficulties in precisely measuring vitamin K levels
9 from the small amounts of serum collected. Because animals had free access to food containing
10 vitamin K_3, the vitamin K levels might have differed among rats. This might be why we detected a
11 correlation between postoperative Gla-OC levels and mechanical properties, but failed to detect a
12 significant difference in the mechanical properties among the PTH_{1-34}, PTH_{1-34} + warfarin, and
13 PTH_{1-34} + vitamin K groups. Second, the number of rats studied (n = 5 per group for biomechanical
14 testing) may be insufficient to detect "small" effects of vitamin K_2 on the mechanical properties of
15 the osteotomized femur. However, the power analysis suggests that n=34 rats per groups would be
16 necessary to detect significant difference in bending strength and such larger studies would not
17 alter the major conclusions in this study. Third, we did not evaluate the effect of other Gla-proteins,
18 such as Matrix Gla protein (MGP) on bone repair. MGP is another major Gla-protein in bone and
19 cartilage. While the function of MGP is not yet fully understood, it is thought to regulate the
20 development of cartilage and to inhibit mineralization. Therefore, the involvement of MGP in
21 bone repair requires further investigation.

22 In summary, we demonstrated that PTH_{1-34} therapy cannot fully exert its effect on bone repair
23 under conditions of vitamin K insufficiency in rats. This finding suggests that PTH_{1-34} therapy is

less effective for enhancing bone repair in patients with a low dietary vitamin K intake or patients using the vitamin K antagonist warfarin as an anticoagulant therapy. Conversely, concomitant use of vitamin K_2 with PTH_{1-34} should improve mechanical recovery of fractured bone by regulating the growth and maturation of hydroxyapatite crystal especially in patients with a low dietary vitamin K intake.

Disclosure

All authors state that they have no conflict of interest.

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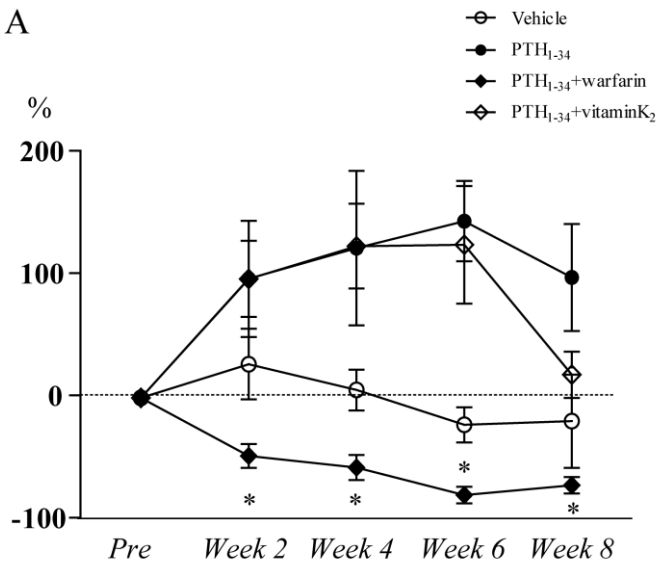
Figure legends

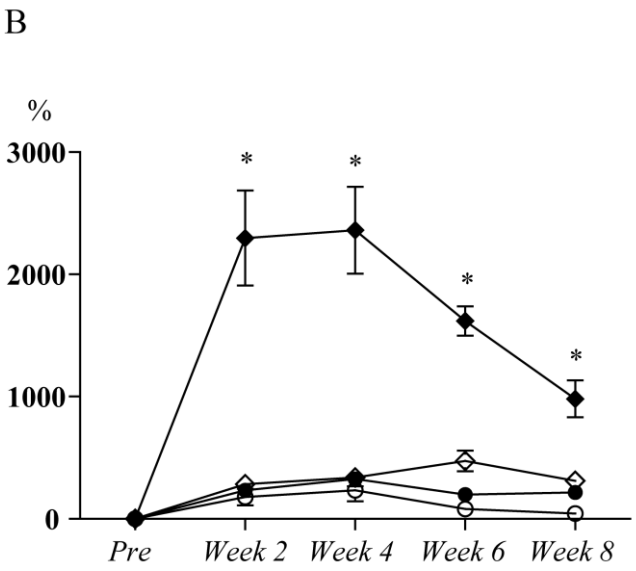
Fig.1. Percent change in Gla-OC (A) and Glu-OC (B) after osteotomy. (A) In the vehicle group, Gla-OC increased 18% from the baseline at week 2 and decreased thereafter. PTH_{1-34} increased 5-fold more in the Gla-OC group compared with the vehicle group at week 2 and the high Gla-OC level was maintained at week 6. Warfarin cancelled the effect of PTH_{1-34} and further decreased Gla-OC to lower than baseline level throughout the observation period. Administration of vitamin K_2 did not have additive effects with Gla-OC for PTH_{1-34} therapy. (B) Glu-OC increased by 100%-200% after osteotomy in all groups except in the PTH_{1-34} + warfarin group. Glu-OC markedly increased in PTH_{1-34} + warfarin group. Values shown are mean ± SEM (n=6). * $P < 0.05$ vs. PTH_{1-34} group.

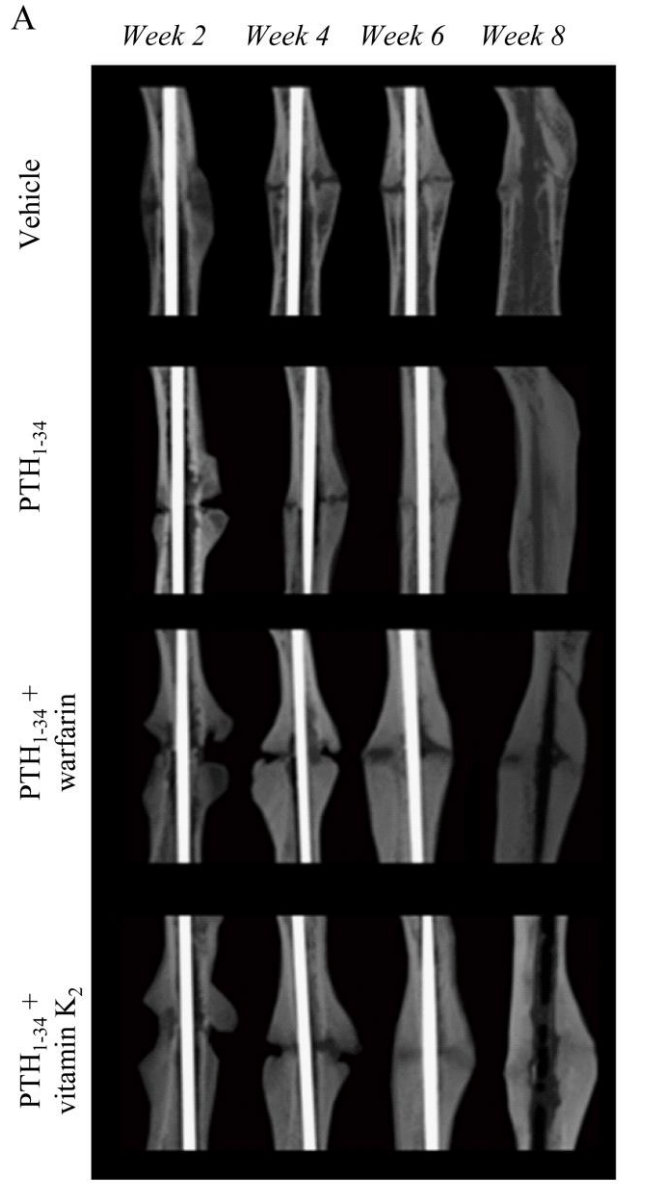
Fig. 2. Effect of vitamin K insufficiency or supplementation on the efficacy of PTH_{1-34} therapy for radiographic healing of osteotomized femurs. (A) Longitudinal 2D-reconstructed micro-CT images of representative specimens in each group are shown. (B) Mineralized bone volume (BV_{callus}) and (C) volumetric bone mineral density ($mBMD_{callus}$) of the callus at 8 weeks postsurgery were calculated from the micro-CT data. PTH_{1-34} groups had higher BV_{callus} and $mBMD_{callus}$ than the vehicle group. Warfarin tended to blunt the efficacy of PTH_{1-34} therapy in terms of BV_{callus} and $mBMD_{callus}$. Values shown are mean ± SEM (n=10). * $P < 0.05$ vs. vehicle group.

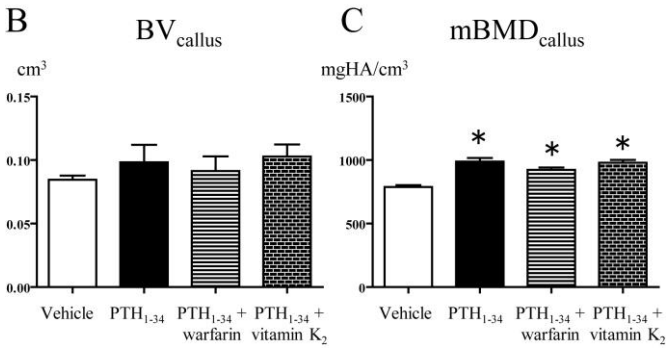
Fig. 3. Histology and histomorphometry of the callus. Longitudinal sections of the osteotomized femurs at 8 weeks postsurgery with Villanueva bone staining were observed by light microscopy (A-H) and under epifluorescent light (I-L). A-D and E-H are low and high

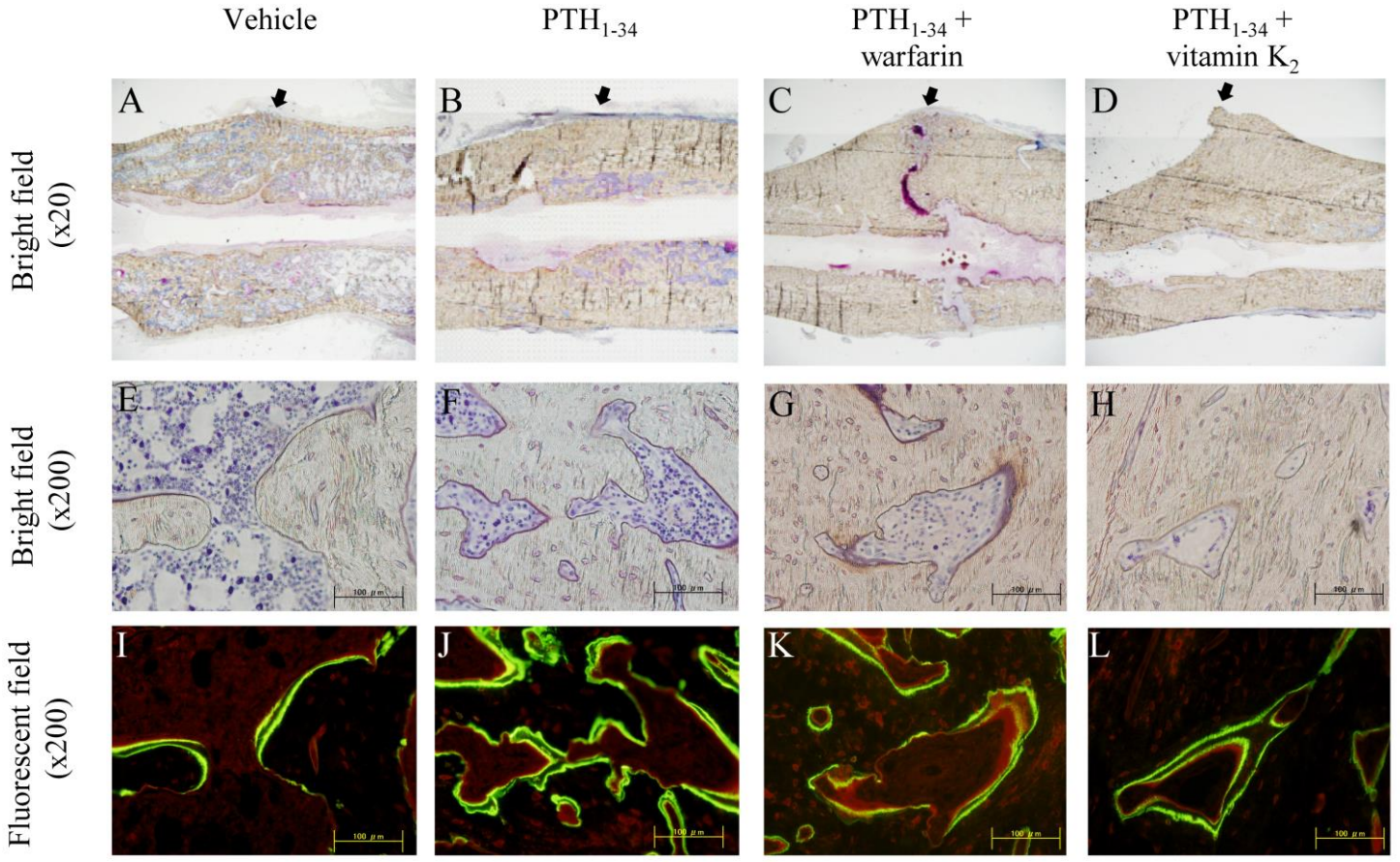
magnification micrographs of the callus, respectively. Osteoids, shown as red-purple in brightfield images or red in epifluorescent light images, were abundant in the PTH_{1-34} and vitamin K groups. Bone histomorphometry data, including (M) bone volume of tissue volume (BV/TV), (N) osteoid volume of bone volume (OV/BV), (O) osteoblast number (N.Ob/BS), (P) osteoblast surface (Ob/BS), (Q) mineralizing surface (MS/BS), (R) mineral apposition rate (MAR), and (S) bone formation rate of bone surface (BFR/BS), are shown. Bars = 100 μm. Values shown are mean ± SEM (n=5). *: $P < 0.05$ vs. vehicle group. **: $P < 0.05$ vs. PTH_{1-34} group.

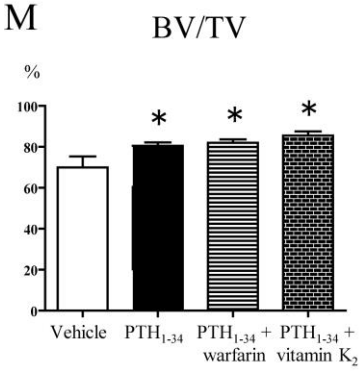

A
Vehicle
PTH1-34
PTH1-34+warfarin
PTH1-34+vitaminK2
%
200
100
0
-100
*
*
*
*
Pre
Week 2
Week 4
Week 6
Week 8

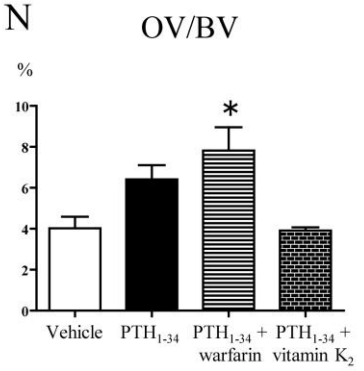

B
%
3000
2000
1000
0
*
*
*
*
Pre
Week 2
Week 4
Week 6
Week 8

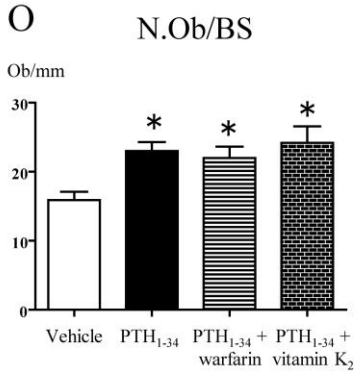

A
Week 2
Week 4
Week 6
Week 8
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2

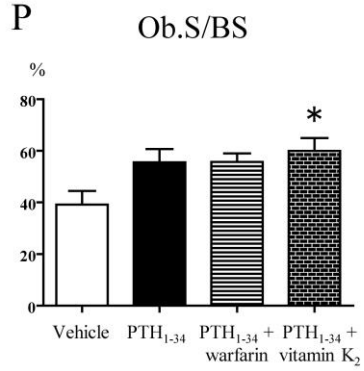

B
BVcallus
cm3
0.15
0.10
0.05
0.00
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2
C
mBMDcallus
mgHA/cm3
1500
1000
500
0
*
*
*
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2

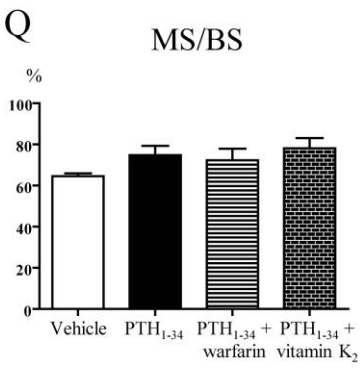

Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2
Bright field (x20)
Bright field (x200)
Fluorescent field (x200)
A
B
C
D
E
F
G
H
I
J
K
L

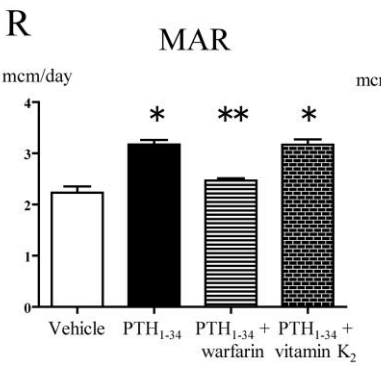

M
BV/TV
%
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2


N
OV/BV
%
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2


O
N.Ob/BS
Ob/mm
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2


P
Ob.S/BS
%
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2


Q
MS/BS
%
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2


R
MAR
mcm/day
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2

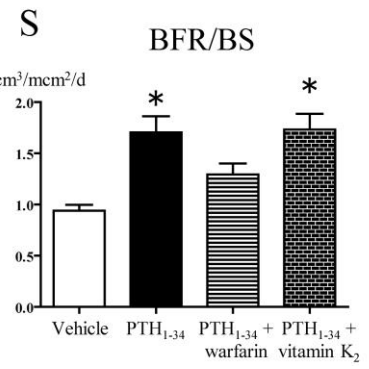

S
BFR/BS
mcm3/mcm2/d
Vehicle
PTH1-34
PTH1-34 + warfarin
PTH1-34 + vitamin K2

Table 1. Biomechanical properties of the osteotomized femurs at 8-week postsurgery.

	Vehicle	PTH_{1-34}	PTH_{1-34} warfarin	PTH_{1-34} vitamin K_2
Ultimate stress (N)	74.6 ± 8.8	180.7 ± 37.9	97.4 ± 33.4	265.9 ± 42.9*
Stiffness (N/mm)	20.0 ± 5.2	34.9 ± 6.0	21.4 ± 8.4	46.3 ± 4.4*
Elastic modulus (MPa)	139.9 ± 36.5	390.9 ± 126.1	188.5 ± 62.9	517.2 ± 89.0*
Bending strength (MPa)	10.0 ± 1.8	34.0 ± 10.0	15.3 ± 4.8	46.1 ± 5.6*

Values are mean ± SEM. n=5 for each group

*: $p<0.05$ vs. vehicle

Table 2: Correlations between 8-week mechanical properties of osteotomized femurs and postoperative serum Gla-OC levels

	Ultimate load	Stiffness	Elastic modulus	Bending strength
Gla-OC *Week 2*	0.6348 p=0.015*	0.4125 p=0.142	0.5050 p=0.061	0.7695 p=0.0013*
Gla-OC *Week 4*	0.7367 p=0.003*	0.6467 p=0.012*	0.6454 p=0.013*	0.6918 p=0.0061*
Gla-OC *Week 6*	0.7339 p=0.003*	0.6333 p=0.015*	0.6365 p=0.014*	0.7764 p=0.0011*
Gla-OC *Week 8*	0.2689 p=0.352	0.2668 p=0.357	0.5052 p=0.062	0.4603 p=0.098

Upper; Peason r, Lower; p value.

* indicates statistically significant correlation $p < 0.05$