



# Effect of parathyroid hormone on healing in osteoporotic fractures via a phospholipase C-independent pathway

Wei-Long Li<sup>1,\*</sup>, Xiao Yu<sup>2,\*</sup>, Zhi-Ping Huang<sup>3</sup>  
and Qing-Jiang Pang<sup>2</sup>

## Abstract

**Objective:** This study was performed to investigate the effect of parathyroid hormone (PTH) on healing in osteoporotic fractures via a phospholipase C (PLC)-independent pathway and explore the mechanism of PTH-mediated bone formation.

**Methods:** Ninety-six 12-week-old C57BL/6J female mice underwent bilateral ovariectomy. One month later, the lower third of the femur was fractured and the mice were treated using saline, PTH(1-28), PTH(1-34), zoledronic acid (ZA), PTH(1-28)+ZA, and PTH(1-34)+ZA. The mice were killed at weeks 2 and 4 in each group. Biomechanical testing and micro-computed tomography were performed.

**Results:** The formation and strength of the callus increased in all but the saline group. The mice treated with PTH(1-34) showed a significantly higher ultimate bending force, bending rigidity, bone mineral density, percent bone volume, and trabecular thickness than those treated with PTH(1-28). The PTH(1-34)+ZA group demonstrated the greatest improvements in the ultimate bending force, bending rigidity, bone mineral density, and relative bone volume.

**Conclusions:** PTH can promote fracture healing and callus hardness in ovariectomized mice by increasing callus formation and reconstructing trabecular bone via a PLC-independent pathway. PTH combined with ZA has a cumulative effect on the healing of fractures in ovariectomized mice.

## Keywords

Parathyroid hormone, signaling pathway, zoledronic acid, osteoporotic fracture

Date received: 31 October 2016; accepted: 4 April 2017

<sup>1</sup>Ningbo University School of Medicine, Ningbo, China

<sup>2</sup>Department of Orthopedics, Ningbo No. 2 Hospital, Ningbo, China

<sup>3</sup>Department of Spinal Surgery, Nanfang Hospital, Southern University, Guangzhou, China

\*These authors contributed equally to this work.

## Corresponding author:

Qing-Jiang Pang, Department of Orthopedics, Ningbo No. 2 Hospital, Ningbo, Zhejiang 315010, China.

Email: pqjey@sina.com



## Introduction

Parathyroid hormone (PTH) promotes bone formation<sup>1</sup> and plays a critical role in maintaining the balance of calcium and phosphorus metabolism and regulating bone synthesis and catabolism. In clinical treatment, PTH has been proven to have a reliable anti-osteoporosis effect; it is currently among the first-choice anti-osteoporosis drugs.

Experimental studies in animals and humans have demonstrated that intermittent administration of low doses of PTH by daily subcutaneous injection effectively stimulates cancellous bone formation, whereas continuous high-dose application of the hormone promotes bone resorption. Therefore, achieving the desired effect of PTH requires careful dosage, and the optimal regimen has not yet been fully elucidated.<sup>2</sup> Whether PTH can promote fracture healing to accelerate rehabilitation of patients with fractures has been widely researched. Previous studies have shown that different doses of PTH can lead to different effects because binding of PTH to the PTH receptor activates different signaling pathways. It is now clear that the biological role of PTH is mainly achieved through activation of three signaling pathways: the cyclic AMP/protein kinase A (cAMP/PKA), phospholipase C/protein kinase C (PLC/PKC), and non-PLC/PKC pathways.<sup>3</sup>

However, the roles of PTH signaling pathways in bone are complex, and the specific role of the non-PLC/PKC signaling pathway in bone formation is not well understood. Its mechanism in bone metabolism requires further study.

The aims of this study were to elucidate the effects of PTH on osteoporotic fracture healing via the non-PLC/PKC signaling pathway and the mechanism of PTH in promoting bone formation. This information will help in the development of PTH-based bone-formation drugs with higher efficacy and fewer side effects.

## Methods

### *Experimental animals*

In total, 110 12-week-old C57BL/6 female mice purchased from the Guangzhou Animal Experimental Center were used in this study. The average weight was 19 to 20 g. All mice were kept in their cages for a 1-week acclimatization period. An osteoporotic fracture model was then established at 13 weeks of age. During the experiment, the animals were handled based on Guidance Opinion about Kindly Treating Animals, released by the Ministry of Science and Technology in 2015.

### *Animal model*

The osteoporosis model was established via a previously described protocol of bilateral ovariectomy (OVX).<sup>4</sup> Penicillin was injected intraperitoneally for 3 consecutive days. Four weeks after OVX, all mice received inhalational anesthesia using isoflurane gas (300 mL/min), then underwent an incision on the left thigh and blunt separation of the muscle and soft tissue to expose the left femur. Intramedullary fixation of the femur was performed by reverse insertion of a needle ( $\varphi=0.45$  mm) from the knee. A transverse osteotomy was then made at the distal one-third of the left femur. Penicillin was injected intraperitoneally for 3 consecutive days. Wound healing was routinely monitored.

### *Pharmaceutical treatment*

After the fracture operation, all 96 mice were randomly divided into 6 groups. The following pharmaceutical interventions (subcutaneous injections) commenced from postoperative day 3: the control group received an equivalent dose of vehicle; the PTH(1-28) group received PTH(1-28) at 400  $\mu$ g/kg five times a week for 4 consecutive weeks; the PTH(1-34) group received PTH(1-34) at 40  $\mu$ g/kg five times a week for

4 consecutive weeks; the zoledronic acid (ZA) group received a bolus dose of ZA at 100 µg/kg; the PTH(1-28)+ZA group received PTH(1-28) at 400 µg/kg five times a week for 4 consecutive weeks plus a bolus dose of ZA at 100 µg/kg; and the PTH(1-34)+ZA group received PTH(1-34) at 40 µg/kg five times a week for 4 consecutive weeks plus a bolus dose of ZA at 100 µg/kg.<sup>5,6</sup> The PTH was sourced from Bachem (Torrance, CA), and the ZA was sourced from Novartis Pharma AG (Basel, Switzerland).

### Specimen collection

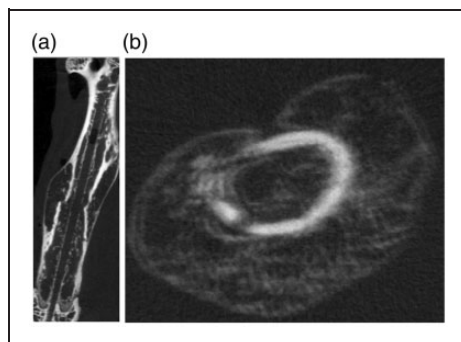
The mice were killed at weeks 2 and 4 after fracture, and the left femur was collected for analysis.

### Micro-computed tomography examination

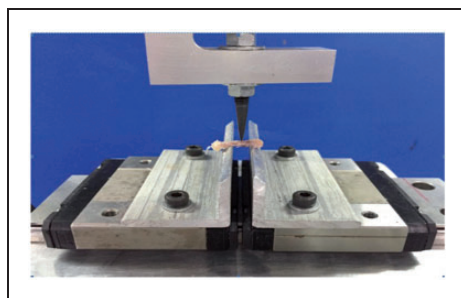
The soft tissue and muscles surrounding the fractured femurs were carefully peeled, and the needle was removed. These specimens were immersed in 10% formalin for 48 hours, then removed and stored in 70% ethanol at 4°C. The fractured femurs were scanned using a micro-computed tomography (micro-CT) system (SkyScan 1176; Bruker, Billerica, MA) while wrapped in moist tissue and placed in a sample scanning holder (Figure 1). The long axis of the femur was aligned with the long axis of the scanner. The scanning system was set to 50 kV, 98 µA, and a 9-µm isotropic voxel size. After the scan, the regions showing new callus formation were selected using micro-CT software, and the following parameters were quantified: bone mineral density (BMD), bone volume (BV), percent bone volume (BV/TV), mean trabecular thickness (Tb.Th), and structural model index.

### Biomechanical testing

Following micro-CT scanning, the left femur underwent biomechanical testing at



**Figure 1.** Micro-computed tomography findings. (a) Coronal and (b) cross section of the left femur.



**Figure 2.** Femoral biomechanical three-point bending test.

weeks 2 and 4 after fracture (Figure 2). Three-point bending on the fractured calluses was performed using a commercial material testing system ElectroPuls (Instron E1000; Instron, Norwood, MA). During the test, the span of the fulcrum was set at 6 mm, and the load was applied vertically at the midpoint of the callus at a speed of 2 mm/min. The ultimate bending force was recorded at the callus breakage, and the bending rigidity was calculated according to the force and displacement curves.

### Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analyses were performed using SPSS 20.0 (IBM Corp.,

Armonk, NY). One-way analysis of variance was used to analyze mean values in each treatment group. If differences were observed between the groups, the least significant difference test was performed ( $\alpha = 0.05$ ).

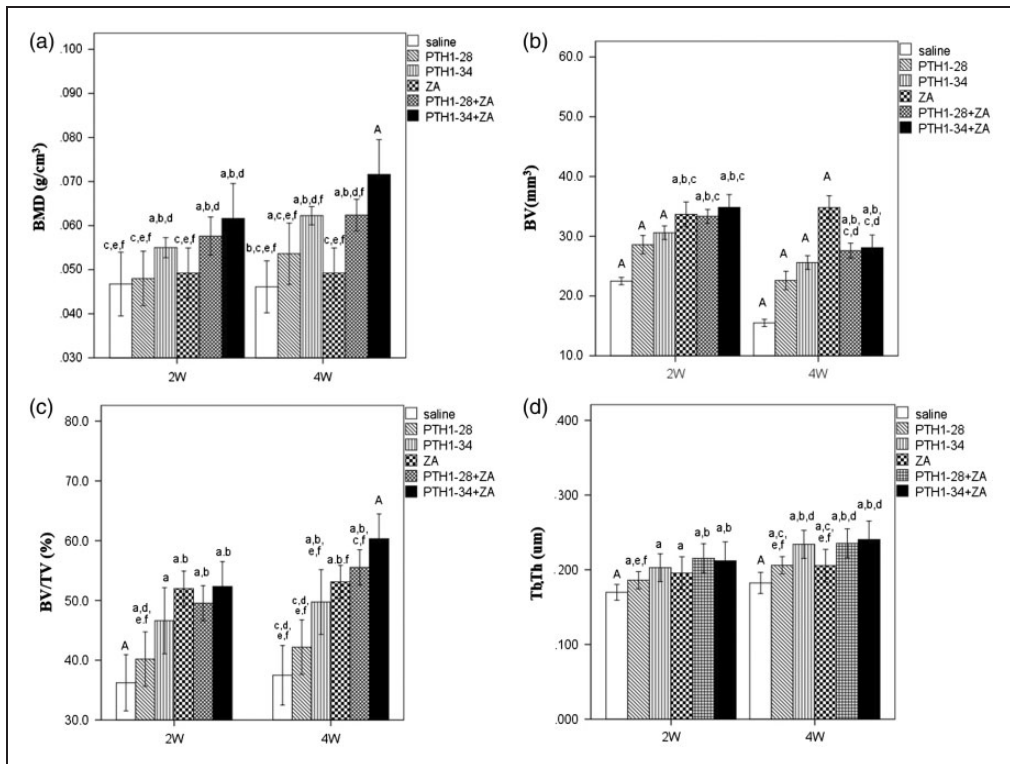
## Results

### Animals

Fourteen mice were excluded from the analysis due to infection, anesthesia, or displacement of the intramedullary fixation after fracture. Eight mice were left for evaluation in each group at each observation time.

### Micro-CT evaluation

Statistically significant differences in BMD, BV, BV/TV, and Tb.Th were observed among the groups ( $P \leq 0.05$ ), while no significant difference in the structural model index was observed. The changes in BMD in each treatment group at weeks 2 and 4 are shown in Figure 3(a). The BMD was significantly higher in the PTH(1-34) than PTH(1-28) group at week 2 ( $0.055 \pm 0.0027$ ) and week 4 ( $0.062 \pm 0.002$ ) ( $P < 0.05$ ). The BMD was not significantly different between the PTH(1-34)+ZA group and the other groups at week 2 ( $0.062 \pm 0.010$ ); however, the BMD was significantly



**Figure 3.** Changes in parameters among the study groups (n = 8/group). (a) Bone mineral density (BMD). (b) Bone volume (BV). (c) Percent bone volume (BV/TV). (d) Mean trabecular thickness (Tb.Th). (a–d) a:  $P < 0.05$  vs. normal saline; b:  $P < 0.05$  vs. PTH(1-28) group; c:  $P < 0.05$  vs. PTH(1-34) group; d:  $P < 0.05$  vs. ZA group; e:  $P < 0.05$  vs. PTH(1-28)+ZA group; f:  $P < 0.05$  vs. PTH(1-34)+ZA group. PTH, parathyroid hormone; ZA, zoledronic acid.

higher in the PTH(1-34)+ZA group than in the other groups at week 4 ( $0.072 \pm 0.009$ ) ( $P < 0.05$ ).

The changes in BV, BV/TV, and Tb.Th in each group at weeks 2 and 4 are shown in Figure 3(b)-(d). The BV/TV was not significantly different between the PTH(1-34) and PTH(1-28) groups at week 2 ( $40.25 \pm 5.5$  vs.  $46.6 \pm 6.62$ , respectively); however, the BV/TV was significantly higher in the PTH(1-34) than PTH(1-28) group at week 4 ( $P < 0.05$ ). The Tb.Th was not significantly different between the PTH(1-34) and PTH(1-28) groups. During the 4-week observation period, PTH(1-34) had a significantly stronger effect on BV than did PTH(1-28) ( $P < 0.05$ ). At week 2, the BV was significantly higher in the PTH(1-34)+ZA and ZA groups than in the groups treated with PTH alone ( $P < 0.05$ ). At week 4, the ZA group exhibited the highest BV ( $P < 0.05$ ). At week 2, the BV/TV in the PTH(1-34)+ZA group was only higher than that in the control and PTH(1-28) groups ( $P < 0.05$ ), while at week 4, the PTH(1-34)+ZA group demonstrated the highest BV/TV ( $P < 0.05$ ). At weeks 2 and 4, no significant differences in Tb.Th were observed between the PTH(1-34) and PTH(1-34)+ZA groups.

### Biomechanical testing

The samples underwent biomechanical testing after micro-CT scanning. The results are presented as the ultimate bending force and bending rigidity. As shown in Tables 1 and 2, the least significant difference test was performed when the two indices were demonstrably different between groups. At week 4, the PTH(1-34)+ZA group showed a 1.3-fold and 2.2-fold higher bending rigidity and 1.2-fold and 2.0-fold higher ultimate bending force than the PTH(1-34) and ZA groups, respectively. Moreover, the PTH(1-34) group demonstrated a 1.2-fold and 1.3-fold higher bending rigidity and

**Table 1.** Ultimate bending force and bending rigidity at week 2 in each group.

Week 2	Bending rigidity (N/m)	Ultimate bending force (N)
Normal saline	$6.7 \pm 0.9$	$3.7 \pm 0.5$
PTH(1-28)	$8.6 \pm 0.7$	$4.3 \pm 0.5$
PTH(1-34)	$10.1 \pm 0.8$	$5.0 \pm 0.7$
ZA	$9.2 \pm 0.9$	$4.5 \pm 0.7$
PTH(1-28)+ZA	$11.1 \pm 1.3$	$5.7 \pm 0.6$
PTH(1-34)+ZA	$11.6 \pm 1.1$	$6.1 \pm 0.7$
F value	27.892	17.229
P value	0.000	0.000

PTH, parathyroid hormone; ZA, zoledronic acid

**Table 2.** Ultimate bending force and bending rigidity at week 4 in each group.

Week 4	Bending rigidity (N/m)	Ultimate bending force (N)
Normal saline	$9.0 \pm 2.8$	$4.7 \pm 0.45$
PTH(1-28)	$14.1 \pm 2.7$	$7.0 \pm 0.98$
PTH(1-34)	$16.9 \pm 2.8$	$8.9 \pm 1.1$
ZA	$9.9 \pm 3.3$	$5.4 \pm 1.2$
PTH(1-28)+ZA	$18.9 \pm 2.2$	$9.1 \pm 1.2$
PTH(1-34)+ZA	$22.1 \pm 2.8$	$10.8 \pm 1.5$
F value	27.226	32.947
P value	0.000	0.000

PTH, parathyroid hormone; ZA, zoledronic acid

ultimate bending force than the PTH(1-28) group, respectively.

### Discussion

Fracture healing is a complex process, and factors that influence this process include the local blood supply, age, health status, and iatrogenic factors.<sup>7</sup> The rate of nonunion or delayed union of fractures is about 5% to 10% and reaches 20% in patients with osteoporosis.<sup>8</sup> PTH not only regulates the formation, transformation, proliferation, and apoptosis of osteoblasts but also

promotes the bone resorption function of osteoclasts; it is also an important regulatory hormone of calcium metabolism *in vivo*. PTH regulates osteoblasts by binding to PTH receptor I on the cell surface, which can activate multiple downstream signaling pathways. Moreover, PTH indirectly regulates osteoclasts via its action on osteoblasts.<sup>9</sup> Jouishomme et al.<sup>10</sup> reported that PTH(29-32) activated PKC on the cell membrane of ROS17/2.8 cells, while PTH(1-31) did not have this effect. This finding indicates that the non-PLC/PKC signaling pathway is relevant to the PTH(29-32) segment. However, the specific signaling mediator molecules and corresponding biological mechanism of the non-PLC/PKC pathway are not well understood. McDonald et al.<sup>11</sup> found that bolus ZA treatment (100 µg/kg) increased the hard callus bone mineral content more than weekly ZA treatment (five weekly doses of 20 µg/kg). However, regardless of the method of delivery, there is no evidence that ZA can promote complete fracture healing. Some studies<sup>12,13</sup> have shown that ZA does not delay endochondral fracture repair and allows hard callus remodeling to proceed in the long term, albeit more slowly than normal fracture healing. Moreover, some scholars<sup>14</sup> have found that PTH combined with bisphosphonates had no significant effect on osteoporosis. However, the doses and injection methods in these studies were inconsistent. PTH combined with ZA was recently shown to have stronger effects on fracture healing than either monotherapy after OVX in rats.<sup>15</sup> The ZA was administered in a small dose via weekly injections, and PTH was injected three times a week. According to other studies,<sup>5,6</sup> we explored the non-PLC/PKC signaling pathway and observed whether it has a stronger effect on bone formation by using an optimal dose of PTH [PTH(1-34) at 40 µg/kg five times a week] and ZA (bolus dose of 100 µg/kg).

Different signaling-selective PTH peptide analogs and commonly used bisphosphonates were compared with regard to their mechanism of action in bone tissue repair to provide more information on the non-PLC/PKC signaling pathway. Bilateral OVX, which results in a decline in the estrogen level, is a classic method by which to establish osteoporosis in animal models. In the past, femoral fracture in experimental animals was produced manually, but the operation was difficult. Since then, specialized equipment to establish femoral fractures in mice has been designed.<sup>16</sup> Despite its high repeatability, the operation is more cumbersome. Furuta et al.<sup>17</sup> used a wire saw to create transverse fractures in the femurs of male mice, and the fractures were directly visualized and fixed with intramedullary nails. The fixation strength of osteoporotic fractures of middle-aged female mice has yet to be verified. In the present study, the method used to fracture the lower third of the femur under direct visualization and fixation with an intramedullary nail in conjunction with OVX has the characteristics of reliable fixation, technical simplicity, and repeatability.

Micro-CT examination and biomechanical testing were performed on the callus to study the fracture healing in each experimental group. PTH(1-34) treatment had a stronger effect on BMD, BV, BV/TV, and Tb.Th than PTH(1-28) treatment. This indicates that while PTH(1-28) activates the cAMP/PKA and PLC/PKC signaling pathways, PTH(1-34) further increases the number of bony trabeculae and strength of the callus and promotes healing of osteoporotic fractures through its action on the non-PLC/PKC signaling pathway. Among all treatment groups, PTH(1-34)+ZA treatment had the strongest effect on BMD, BV/TV, Tb.Th, ultimate bending force, and bending rigidity. In addition, at week 2, the PTH(1-34)+ZA and ZA groups showed a higher BV than those groups treated with a



single drug. At week 4, the ZA group demonstrated the highest BV, which could be explained by the antiresorptive properties of ZA. The PTH(1-34)+ZA group also demonstrated a high BV, but it was lower than that in the ZA group, indicating that PTH can promote cancellous bone formation in the bone callus and remodeling of trabecular bone. At week 2, PTH(1-34)+ZA and ZA treatment had a similar effect on BV/TV; however, the PTH(1-34)+ZA group had the highest BV/TV at week 4. PTH(1-34)+ZA treatment produced the most bone mass, indicating that ZA may inhibit bone resorption while PTH promotes bone formation. The combination of the two drugs not only decreases the loss of the bone mass of the callus but can also generate new bone tissue. Consequently, this group showed the maximum increase in bone mass. The PTH(1-34)+ZA group had the greatest callus intensity, which may be dependent on increasing the amount of callus to improve early callus strength, while the later callus strength is mainly dependent on callus remodeling. All findings of this study show that PTH(1-34) combined with ZA can increase the callus quantity, enhance callus remodeling, improve bone morphology, and promote bone growth via multiple signaling pathways in an osteoporotic fracture animal model.

In summary, PTH can promote fracture healing after OVX in mice by increasing the amount of callus and remodeling the callus via a non-PLC/PKC signaling pathway and increasing the callus hardness. Moreover, PTH combined with ZA had synergistic effects on fracture healing after OVX in mice. However, the observation time of this study was only 4 weeks; a longer period of observation is required to more fully understand the effects of monotherapy or combined therapy. In addition, these results were obtained in mice and may not translate fully to the human body. Clinical trials are required to prove the effectiveness of this

treatment in patients. Additional cellular or molecular studies may cast more light on the mechanism behind these therapies. PTH combined with ZA has a synergistic effect on fracture healing in mice. Future studies should be performed to explore different doses and different injection frequencies of PTH and ZA for fracture healing.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

### Funding

This study was supported by grants from Zhejiang Medical and Health Science Research Foundation (2015ZDA026), Key subject of Ningbo (2016-55) and Clinical specialty in joint disease of Ningbo (2013-88).

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