

Optimal Timing for Intermittent Administration of Parathyroid Hormone (1-34) for Distraction Osteogenesis in Rabbits

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Abstract

Background

To date, the usefulness of parathyroid hormone (PTH (1-34)) in distraction osteogenesis has been reported in several studies. We aimed to determine the optimal timing of PTH (1-34) administration in a rabbit distraction osteogenesis model.

Methods

The lower hind leg of a Japanese white rabbit was externally fixed, and tibial osteotomy was performed. One week after the osteotomy, bone lengthening was carried out at 0.375 mm/12 h for two weeks. After five weeks, the lower leg bone was collected. Bone mineral density (BMD), peripheral quantitative computed tomography (pQCT), micro-computed tomography (micro-CT), and mechanical tests were performed on the distracted callus. The rabbits were divided into three groups according to the timing of PTH administration: four weeks during the distraction and consolidation phases (group D+C), two weeks of the distraction phase (group D), and the first two weeks of the consolidation phase (group C). A control group (group N) was administered saline for four weeks during the distraction and consolidation phases. Furthermore, to obtain histological findings, lower leg bones were collected from each rabbit at two, three, and four weeks after osteotomy, and tissue sections of the distracted callus were examined histologically.

Results

The BMD was highest in group C and was significantly higher than group D. In pQCT, the total cross-sectional area was significantly higher in groups D+C, D, and C than group N, and the cortical bone area was highest in group C and was significantly higher than group D. In micro-CT, group C had the highest bone mass and number of trabeculae. Regarding the mechanical test, group C had the highest callus failure strength, and this value was significantly higher compared to group N. There was no significant difference between groups D and N. The histological findings revealed that the distracted callus mainly consisted of endochondral ossification in the distraction phase. In the consolidation phase, the chondrocytes were almost absent, and intramembranous ossification was the main type of ossification.

Conclusion

We found that the optimal timing of PTH (1-34) administration is during the consolidation phase, which is mainly characterized by intramembranous ossification.

Background

Distraction osteogenesis is a surgical technique that ensures new bone formation in the distraction gap by adding traction stress to the osteotomy site. This process has three phases: 1) latency phase—the waiting period from osteotomy to the start of distraction, 2) distraction phase—the period of bone lengthening, and 3) consolidation phase—the waiting period for callus maturation after the end of

distraction [1–3] (Fig. 1). Distraction osteogenesis is used in clinical practice to treat various bone defects [4–10]. Other surgical techniques for bone defects include vascularized bone grafts [11] and the Masquelet technique [12–14]. Compared to these techniques, the advantage of distraction osteogenesis is that normal tissue is not sacrificed because bone grafting is unnecessary and special techniques such as microsurgery are not required. On the other hand, the disadvantage is the long period required for bone fusion, during which an external fixator must be worn for an extended period. Clinically, a 1-cm of distracted callus takes one month to mature and heal [15]. In the case of a 10-cm bone defect, an external fixator should be worn for 10 months. Long-term external fixation may increase the burden on daily and social life, economic and mental burden, and risk of infection at the pin insertion site [16–18]. Therefore, the treatment period of distraction osteogenesis needs to be shortened. There are ongoing attempts to combine distraction osteogenesis with other treatment modalities, such as ultrasonic waves [19], extracorporeal shock waves [20], alternating current electrical stimulation [21], dynamization [22], and various bone metabolism regulators and cytokine administration [23–30]. The parathyroid hormone (PTH (1-34)) has been applied clinically to treat osteoporosis and has excellent efficacy in increasing bone mineral density (BMD) and inhibiting bone fractures [31]. The usefulness of PTH (1-34) in fracture healing has been widely studied. In a rat femoral fracture model, PTH (1-34) has been shown to increase BMD, ultimate load, callus volume, callus strength, and promote fracture healing [32–37]. A recent review article also showed that intermittent administration of PTH (1-34) promotes fracture healing in animal studies [38]. Human studies are few and inconsistent with animal studies, but they provide insight into the potential for intermittent PTH (1-34) administration to promote fracture healing.

To date, the usefulness of PTH (1-34) in distraction osteogenesis has been noted only in a few reports [27–30]. Among them, Aleksynien et al. found that callus strength, callus volume, and BMD were not significantly different when PTH (1-34) was administered during the entire distraction and consolidation phases or when administered only during the consolidation phase in a rabbit distraction osteogenesis model [27]. However, no studies have evaluated the optimal timing of PTH (1-34) administration during the distraction and consolidation phases, considering that the type of ossification is different in these two phases [39,40].

This study aimed to determine the optimal administration timing in which PTH (1-34) is most effective in callus formation using a rabbit distraction osteogenesis model. Furthermore, a histological examination of the distracted callus in the distraction and consolidation phases was performed.

Methods

Administration of PTH (1-34)

The PTH (1-34) used in this study was human PTH (1-34), teriparatide acetate (Asahi Kasei Pharma, Tokyo, Japan), which is a sequence of 34 N-terminal amino acids. The molecular weight of teriparatide is 4418 Da. A 10 µg/mL PTH solution was prepared using saline as a solvent, and this was divided into 1-mL containers and cryopreserved at -80°C. The PTH solution was thawed at room temperature

immediately before administration. The amount of solution corresponding to the body weight of each individual rabbit was calculated, and the required amount was subcutaneously injected. The same amount of saline was used in a control group.

Distraction osteogenesis model

The distraction osteogenesis model was based on Maruno's method [29]. For anesthesia, ketamine hydrochloride 15 mg/kg (Sankyo Pharmaceutical Co., Ltd., Tokyo, Japan) was intramuscularly injected, and then pentobarbital 30 mg/kg (Tanabe Pharmaceutical Co., Ltd., Tokyo, Japan) was intravenously administered. A skin incision of approximately 40 mm was made on the medial side of the lower right hind leg to expose the periosteum on the medial aspect of the tibia. Without peeling the periosteum, two half pins with a diameter of 2.0 mm were inserted across the tibiofibular junction at an intermediate distance of 20 mm, and an external fixator for the short tubular bone of humans (Orthofix M-100; Verona, Italy) was attached. The distance between the two central pins was 20 mm, and the bone was cut at 10 mm distal to the tibiofibular junction using a drill hole. During osteotomy, saline was added dropwise to the osteotomy site to prevent heating. The skin was sutured after turning the extender in the direction for shortening to avoid the gap at the osteotomy site. The left hind leg was not treated. The hind legs were loaded immediately after surgery without any restriction. One week after the osteotomy, the bones were distracted by 0.375 mm every 12 hours for two consecutive weeks, according to the method of Little et al. [26]. The amount of bone distraction was 0.75 mm per day, and the total distraction distance was 10.5 mm, corresponding to approximately 9.4% of the total tibial length. Following completion of the two-week extension, the external fixator was removed after the waiting period of four weeks. Eight weeks after the operation, the rabbits were euthanized, and the lower leg bone was collected (Fig. 2).

Animals and test groups

Twenty-eight Japanese white rabbits (Oriental Yeast Co., Ltd., Tokyo, Japan) weighing 2.5 to 2.8 kg were used. PTH (1-34) was subcutaneously injected at a volume of 30 µg/kg every other day. There were four groups included in this study (group D+C, D, C, N), each with seven rabbits. In group D+C, PTH (1-34) was administered for four weeks (two weeks distraction phase + two weeks consolidation phase) from the start of distraction. In group D, PTH (1-34) was administered for two weeks during the distraction phase, and saline was administered for two weeks during the consolidation phase. In group C, saline was administered for two weeks during the distraction phase, and PTH (1-34) was administered for two weeks during the consolidation phase. Group N was the control, and saline was administered for four weeks from the start of distraction.

In addition, to confirm the type of ossification at the distraction site in this experimental model, a histological examination of the distracted callus was performed using three Japanese white rabbits weighing 2.5 to 2.8 kg. The histological examination was performed at the distraction phase (one week

after the start of distraction), at the end of the distraction phase (two weeks after the start of distraction), and at the consolidation phase (one week after the end of distraction).

Measurement methods

The collected lower leg bone was wrapped in saline-soaked gauze and cryopreserved at -80°C until each test was performed. At the time of measurement, the tibia was naturally thawed at room temperature, and various imaging tests were performed, followed by a three-point bending test. The following parameters were measured and compared between groups.

Bone mineral density (BMD)

BMD of the distracted callus was measured by dual-energy X-ray absorptiometry (DXA) for small animals using DCS-600EX-R (Aloka, Japan). BMD was measured 4 mm both proximally and distally from the osteotomy site.

Peripheral quantitative computed tomography (pQCT)

BMD measuring device for small animals, XCT Research SA + pQCT (Stratec Medizintechnik GmbH, Pforzheim, Germany), was used to assess the cross-sectional shapes of the distracted callus. Measurement parameters included total (mm³) and cortical (mm³) areas of the distracted callus.

Micro-computed tomography (micro-CT)

Micro-CT was measured using a high-resolution microfocus X-ray CT scanner, ScanX-mate-A090S (Comscan Techno, Japan), to evaluate the three-dimensional morphology of the cortex and trabecular structure of the distracted callus. Bone volume (BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were measured as parameters of the micro-trabecular structure.

Mechanical testing (three-point bending test)

The three-point bending test was performed using GRAPH-2000E (Shimadzu, Japan). The anterior surface of the tibia faced upwards, whereas the posterior surface faced downwards to allow the center of the distraction osteogenesis to be located in the middle between two support points at 20 mm distance. Then, pressure was applied with a crosshead speed of 2 mm/s at the center of the distracted callus from the anterior tibia surface. The measurement parameter was absorption energy until callus fracture (work to failure (Nmm)).

Histological examination

The lower leg bone collected at each evaluation period was decalcified using formic acid-formalin and was cut into sagittal sections, which were stained using hematoxylin and eosin.

Statistical analysis

For statistical analysis, JMP[®] 15 (SAS Institute Inc., Cary, NC, USA) statistical software was used, with the significance set at $p < 0.05$. Values are expressed as mean \pm standard deviation (SD). Differences between groups were tested by analysis of variance with a Tukey–Kramer honestly significant difference for post hoc analysis.

Ethics

This study conformed to Japan’s “Law concerning the protection and control of animals,” “Standard concerning the breeding and protection of laboratory animals,” “Laboratory Animal Guidelines of Kyorin University,” and other relevant guidelines. The study was conducted at a laboratory of the Department of Orthopedic Surgery, Kyorin University.

Results

Six rabbits were excluded due to loosening of the external fixator pin or fracture during the treatment course, and the remaining 22 rabbits (seven rabbits in group D+C, five rabbits in group D, five rabbits in group C, and five rabbits in group N) were evaluated.

Imaging tests

BMD (mg/cm²) (Table 1)

The BMD (mean \pm SD) of the whole distracted callus by DXA was highest in group C (256 ± 18) and was significantly higher compared with group D (196 ± 15).

Table 1

BMD measured with DXA, and total area and cortical area measured with pQCT

	<i>n</i>	BMD (mg/cm ²)	Total area (mm ³)	Cortical area (mm ³)
Group D+C	7	206 ± 28	76 ± 2 #	25 ± 3
Group D	5	196 ± 15	81 ± 3 #	24 ± 1
Group C	5	256 ± 18 *	76 ± 1 #	28 ± 1 *
Group N	5	239 ± 42	70 ± 1	25 ± 3

*p < 0.05 versus Group D,

#p < 0.05 versus Group N

The values are presented as mean ± standard deviation

pQCT (Table 1)

The total area (mean ± SD) was highest in group D. Compared with group N, groups D+C, D, and C were significantly higher by 9%, 16%, and 9%, respectively. The cortical area (mean ± SD) was highest in group C and was significantly different compared with group D.

Micro-CT (Table 2)

BV/TV (mean ± SD) and Tb.N (mean ± SD) were both highest in group C, and there were significant differences compared with group D. Tb.Sp (mean ± SD) was lowest in group C and showed a significant difference to group D. These differences were confirmed with three-dimensional images of the trabecular bone evaluated with micro-CT (Fig. 3).

Table 2

Micro-architecture of the distracted callus evaluated using micro-CT

	<i>n</i>	BV/TV (%)	Tb.N (1/mm)	Tb.Sp (μ m)
Group D+C	7	44 \pm 14	3.0 \pm 0.8	235 \pm 99
Group D	5	29 \pm 4	2.1 \pm 0.6	383 \pm 156
Group C	5	49 \pm 3 *	3.2 \pm 0.4 *	172 \pm 29 *
Group N	5	49 \pm 13	2.8 \pm 0.2	229 \pm 83

*p < 0.05 versus Group D

The values are presented as mean \pm standard deviation

Mechanical testing (three-point bending test) (Fig. 4).

The work to failure (mean \pm SD) was 471 \pm 55 in group D+C, 427 \pm 99 in group D, 595 \pm 126 in group C, and 313 \pm 48 in group N. The work to failure (mean \pm SD) was highest in group C. Compared with group N, the work to failure for groups C and D+C increased by 90% and 50%, respectively, with statistical significance. There was no significant difference between groups D and N and between groups C and D+C.

Histological examination (Fig. 5)

At the distraction phase, the chondrocytes were abundant in the distracted callus, and the new bone was formed by endochondral ossification. At the end of the distraction phase, two weeks after the start of distraction, the chondrocytes decreased and intramembranous ossification was notable. In the consolidation phase, which began one week after the end of distraction, only a few chondrocytes were identified, and the new bone in the callus distraction part was almost formed by intramembranous ossification.

Discussion

Intermittent administration of PTH (1-34) promotes the differentiation of mesenchymal stem cells into osteoblasts and accelerates bone formation. Studies using ovariectomized cynomolgus monkeys [41–43] have shown that PTH (1-34) administration significantly increases bone mass, improves bone microarchitecture, and increases bone strength in the vertebral body and femoral neck. Furthermore, in a study using human iliac bone biopsy specimens, PTH (1-34) administration showed an increase in total bone mass. Significant improvements in the trabecular structure model index, trabecular connectivity density,

and cortical bone width were observed compared to the placebo group [44]. However, the mechanism by which PTH (1-34) activates bone metabolism predominantly in bone formation remains largely unknown.

To date, research on the usefulness of PTH (1-34) in distraction osteogenesis has been limited. Sheebach et al. used a rat femur extension model with PTH (1-34) administration, 60 µg/kg daily, for a 10-day distraction phase and a 20-day consolidation phase. Following the study, they reported a 58% increase in callus mass, 24% increase in BMD, and 50% increase in maximum point load [28]. Aleksynien et al. used a rabbit tibial distraction model with PTH (1-34) administration, 25 µg/kg daily, for a 10-day distraction phase and a 20-day consolidation phase, and they reported an increase in BMD and bone strength [27]. Recently, by using a rabbit distraction osteogenesis model, we reported an increase in the amount of cortical bone formation and callus width, as well as a significant increase in callus strength after administering 30 µg/kg of PTH (1-34) every other day over a 14-day distraction phase and 35-day consolidation phase [29]. In addition, we have shown that PTH (1-34) administration shortened the callus maturation period by two weeks [30]. Although the PTH (1-34) dose, administration interval, and duration of the distraction and consolidation phases used in our study were different to the study reported by Aleksynien, both emphasized the usefulness of PTH (1-34) for rabbit distraction osteogenesis.

Regarding the optimal timing of PTH (1-34) administration for distraction osteogenesis, Aleksynien et al. reported that administering PTH (1-34) for both distraction and consolidation phases was not superior to PTH (1-34) at only the consolidation phase in the callus volume, BMD, and work to failure [27]. Richards et al. investigated the bone formation in a rabbit tibial distraction osteogenesis model and showed that bone formation was the best in the first half of the consolidation phase, suggesting that this phase may be the optimal time for biological and mechanical interventions for promoting bone regeneration [45]. In a recent distraction osteogenesis study in humans, Wagner et al. reported that administering PTH (1-34) during the consolidation phase raised the rate of regenerated calcification two-fold compared to patients without PTH (1-34) [46]. In our current study, for the first time, PTH (1-34) was administered separately during the distraction and consolidation phases to elucidate the optimal administration timing. Our results showed that BMD, cortical area, and the distracted callus strength were highest in the group that received PTH (1-34) in the consolidation phase only. In addition, the total distracted callus area was significantly higher in all groups that received PTH (1-34) than in the control group regardless of the timing of administration. Except for the group that received PTH (1-34) during the distraction phase only, the distracted callus strength was significantly higher in the group that received PTH (1-34) than in the control group. The increase in the total cross-sectional area of callus was considered to be related to callus strength. However, although the total distracted callus area was highest in the group that received PTH (1-34) in the distraction phase only, the distracted callus strength was not significantly different compared with the control group. This result might have been influenced by the micro-architecture of the distracted callus. In general, cylindrical structures, such as long bones with a larger diameter and longer outer peripheral length, are known to have higher strength on the premise that the amount of the constituent materials is the same [47]. In micro-CT, the distracted callus micro-architecture of the group that received PTH (1-34) in the distraction phase only showed less bone mass and Tb.N and larger Tb.Sp. Therefore, the distracted callus strength was not sufficiently obtained, despite the large total area. In

contrast, in the group that received PTH (1-34) during the consolidation phase only, the total distracted callus area was significantly larger than in the control group with higher bone mass and Tb.N and smaller Tb.Sp, which may lead to the highest strength.

In distraction osteogenesis, a new bone is predominantly formed by endochondral ossification in the distraction phase and by intramembranous ossification in the consolidation phase [39, 40]. However, the ossification form may vary according to the type of experimental animal and protocol. Therefore, the histological evaluations of the distracted callus were also confirmed in this study. Our results were consistent with previous reports, by endochondral ossification being predominant in the distraction phase and intramembranous ossification in the consolidation phase. The callus formed during the healing process after a fracture consists of a mixture of intramembranous and endochondral ossification [48], which is difficult to distinguish with histological examination. In contrast, in the distraction osteogenesis model, type of ossification is reproducible with the phase, such as distraction or consolidation phases. Based on our results that the consolidation phase is the optimal PTH (1-34) administration period, we believe that PTH (1-34) may play a dominant role in intramembranous ossification.

Furthermore, in fracture healing, PTH has been shown to promote the proliferation and differentiation of chondrocytes [49]. Therefore, even in distraction osteogenesis, PTH may also have a promoting effect on chondrocytes during the distraction phase. However, our results do not sufficiently support this conjecture. Accordingly, it is important to consider the difference in chondrogenesis between fracture repair and distraction osteogenesis. In fracture healing, large hematomas, which contain abundant mesenchymal cells and respond to PTH form immediately after fracture, and endochondral bone formation robustly occurs in the first period. On the other hand, in distraction osteogenesis, due to fewer hematomas being present, substantially less cartilage is formed, and its formation is restricted to the early distraction phase. After this phase, it is rapidly resorbed [50]. Therefore, the effect of PTH (1-34) on endochondral ossification during the distraction phase may be difficult to fully achieve in distraction osteogenesis. Later, during the consolidation phase, proliferating osteoprogenitor cells differentiate from osteoblasts into osteocytes, resulting in intramembranous ossification. We consider that PTH (1-34) may have its most significant effect on promoting the differentiation of osteoprogenitor cells during the consolidation phase.

Despite the usefulness of PTH (1-34) for distraction osteogenesis, its application in clinical practice remains a challenge. PTH (1-34) is an expensive drug with a limited lifetime use of two years [51, 52]. Identification of the optimal time when PTH (1-34) acts most effectively on callus formation for distraction osteogenesis in humans will improve patient compliance and cost-effectiveness. Regardless of whether PTH (1-34) was administered during the distraction and consolidation phases or only during the consolidation phase, there was no significant difference in the mechanical and structural properties of the distracted callus. The current findings suggest a clinical implication that will definitely contribute to reduce the PTH (1-34) treatment time in distraction osteogenesis.

This study had several limitations. First, in this work, PTH (1-34) was administered only in the first half of the consolidation phase, the effect of PTH (1-34) administration in the latter half of the consolidation phase remains unknown and should be investigated in the future. Second, considering that the bone anabolic effect on PTH (1-34) differs depending on the administration method [53, 54], the optimal administration approach, including the administration interval and injection site, should be examined in the future. Third, the dose of PTH (1-34) used in this experiment was 30 µg/kg, which is equivalent to the dose of other similar animal experiments (25–60 µg/kg). However, this dose is much higher than that approved by the US Food and Drug Administration for the treatment of osteoporosis patients (0.25–0.5µg/kg/day). The dosage of PTH (1-34) needs to be investigated further for clinical application in humans.

Conclusions

In the rabbit distraction osteogenesis model, administration of PTH (1-34) during the consolidation phase formed a distracted callus with a stronger and better micro-architecture than the distraction phase. Furthermore, PTH (1-34) was shown to act more effectively for the intramembranous ossification during the consolidation phase than for the endochondral ossification during the distraction phase. Determining the optimal timing of PTH (1-34) administration may have a beneficial impact on patient compliance and cost-effectiveness.

Abbreviations

BMD, bone mineral density; BV/TV, bone volume; DXA, dual-energy X-ray absorptiometry; micro-CT, micro-computed tomography; pQCT, peripheral quantitative computed tomography; PTH, parathyroid hormone; SD, standard deviation; Tb.N, trabecular number; Tb.Sp, trabecular separation.

Declarations

- **Ethics approval and consent to participate**

This study conformed to the Japan's "Law concerning the protection and control of animals," "Standard concerning the breeding and protection of laboratory animals," "Laboratory Animal Guidelines of Kyorin University," and other relevant guidelines. It was conducted in a laboratory of the Department of Orthopedic Surgery, Kyorin University.

- **Consent for publication**

Not applicable

• Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

• Competing interests

The authors declare that they have no competing interests

• Authors' contributions

NI, TO, and HM carried out operation. SI conceived of the study design. NI, NH, TM, and SI interpreted the data and drafted the manuscript. NI, NH, TM, and SI were involved in revising the manuscript. All authors read and approved the final manuscript.

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Figures

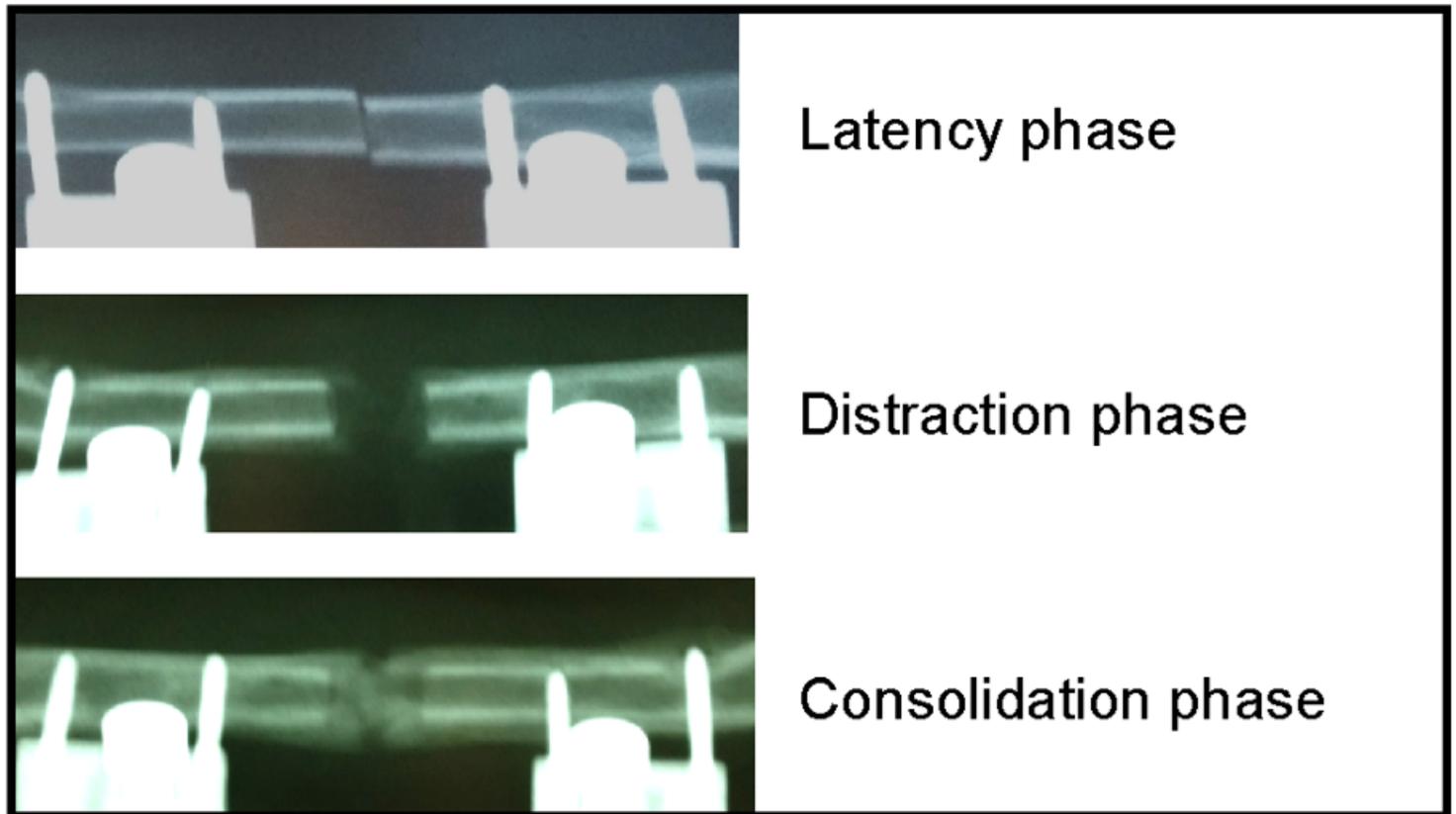


Figure 1

Three phases in distraction osteogenesis There are three phases in the course of distraction osteogenesis (latency, distraction, and consolidation). The latency phase is the waiting period from the time of osteotomy to the start of distraction. The distraction phase is the period in which tension is applied to the callus at a constant rate and rhythm to extend it to the desired length. The consolidation phase is the period in which the callus undergoes mineralization and finally stabilizes.

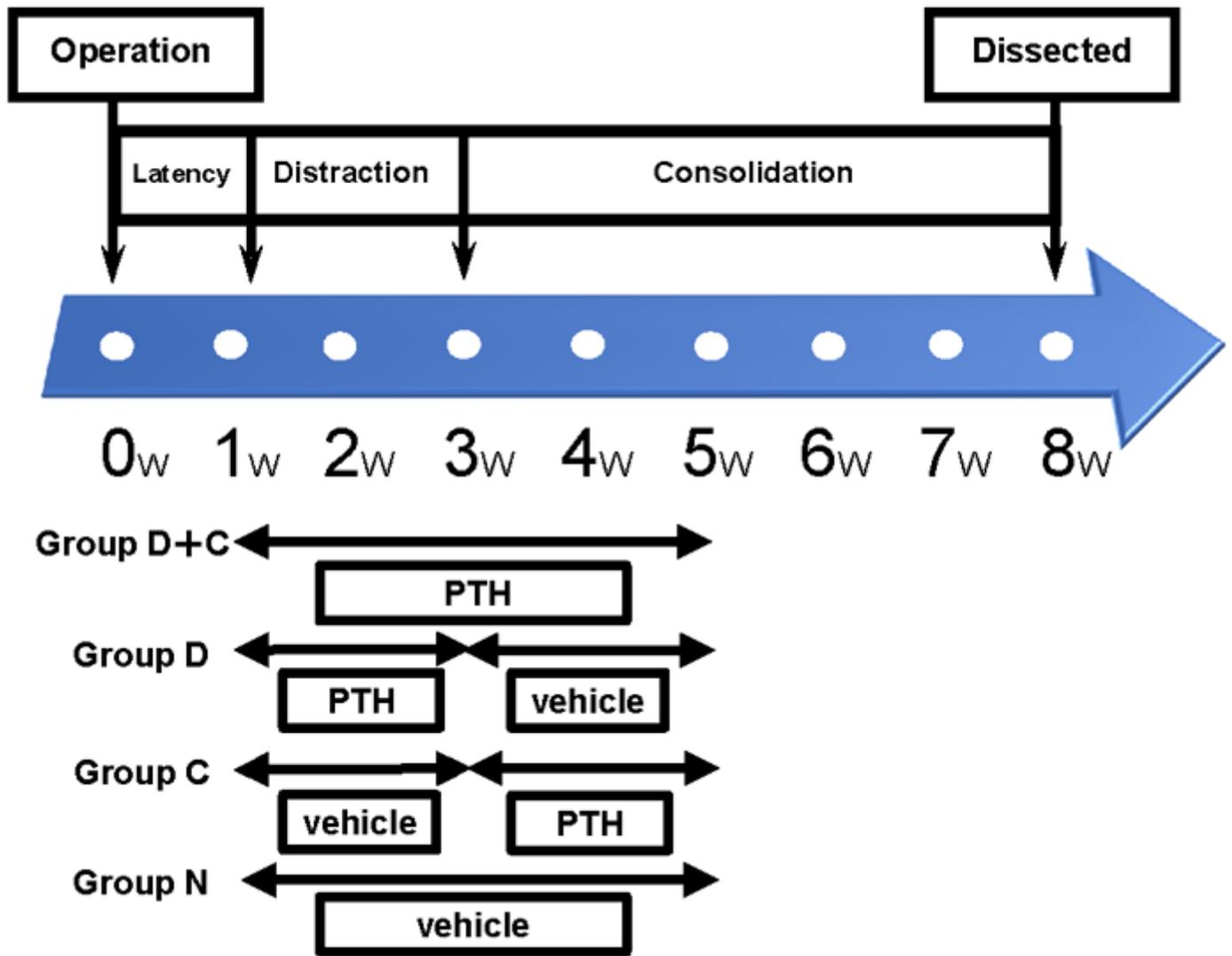


Figure 2

Experimental protocol The one-week latency phase was followed by a two-week distraction phase and a five-week consolidation phase. Group D+C received PTH (1-34) for four weeks from the start of distraction. Group D received PTH (1-34) for two weeks from the start of distraction and saline for the subsequent two weeks. Group C received saline from the start of distraction and PTH (1-34) for the subsequent two weeks, and group N received saline solution for four weeks from the start of distraction. The experimental animals were euthanized at 8 weeks after osteotomy.

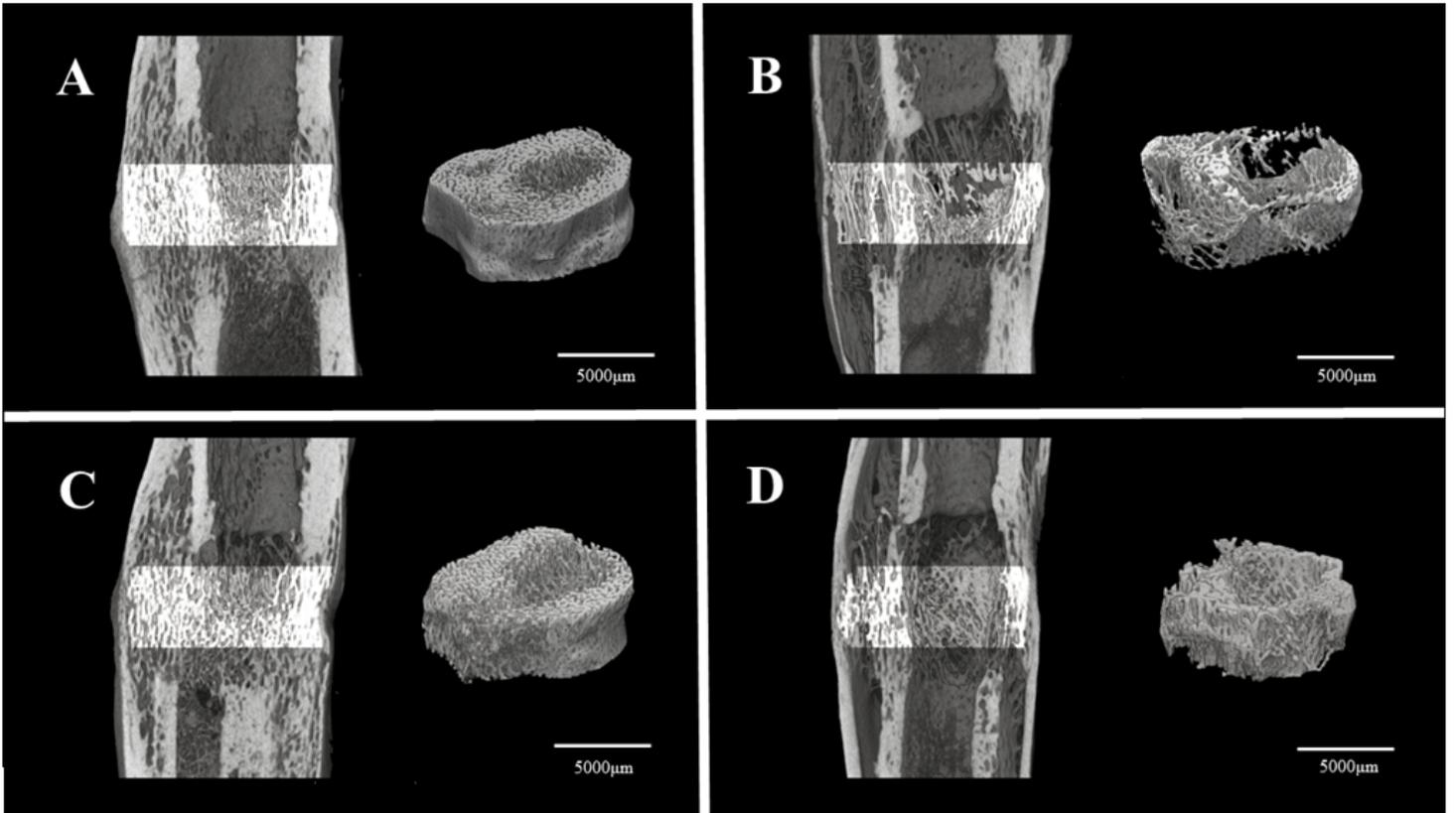


Figure 3

Three-dimensional micro-computed tomography images of the distracted callus Longitudinal (left side) and transverse (right side) sections for a representative specimen. (A) Group D+C; (B) Group D; (C) Group C; (D) Group N

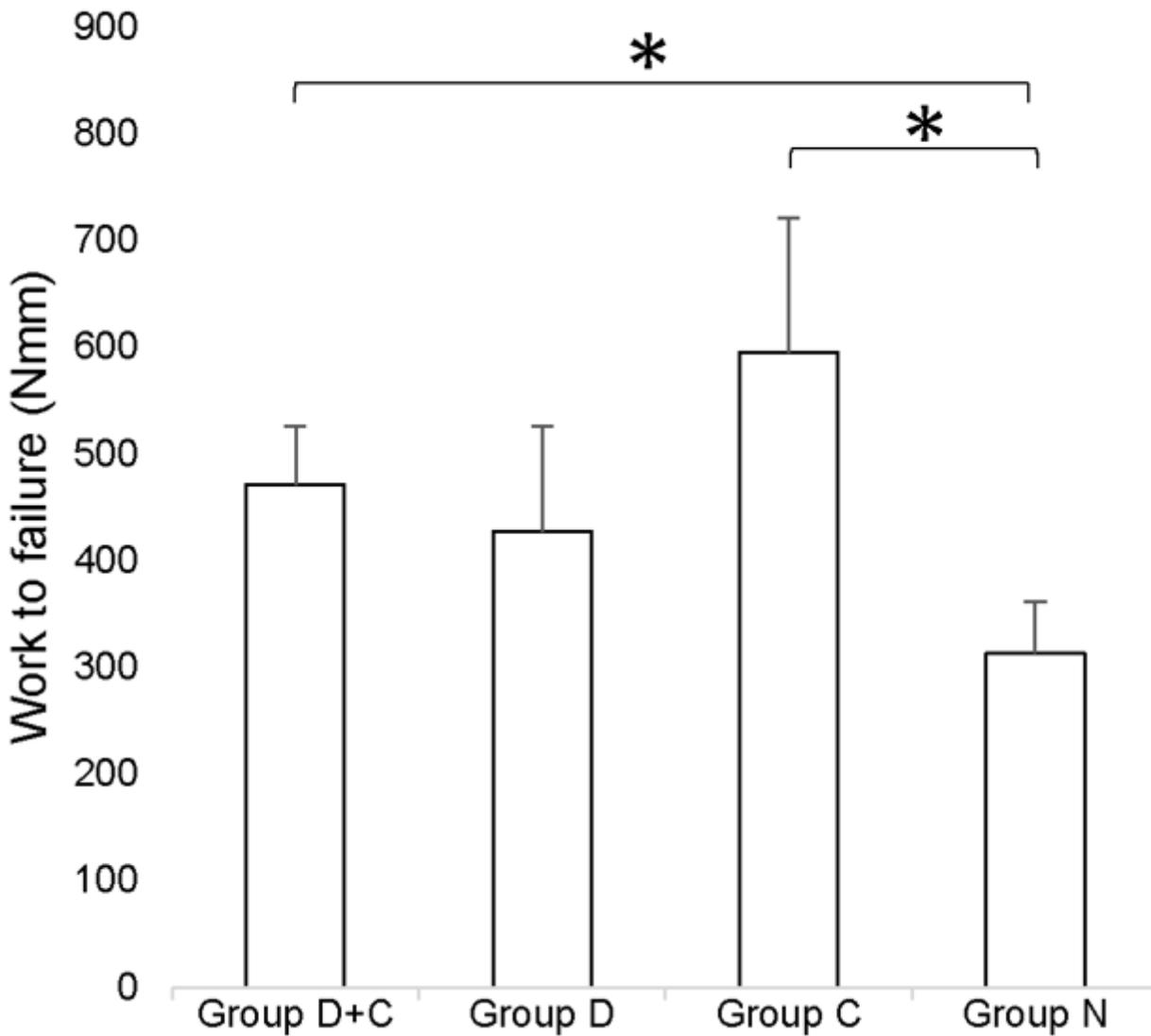


Figure 4

Mechanical properties by three-point bending analysis The measurement parameter was absorption energy until the distracted callus fracture. The work to failure (mean \pm SD) was highest in group C. Compared with group N, the work to failure for groups C and D+C increased by 90% and 50%, respectively, with statistical significance. There was no significant difference between groups D and N and between groups C and D+C. * $p < 0.05$, among four groups.

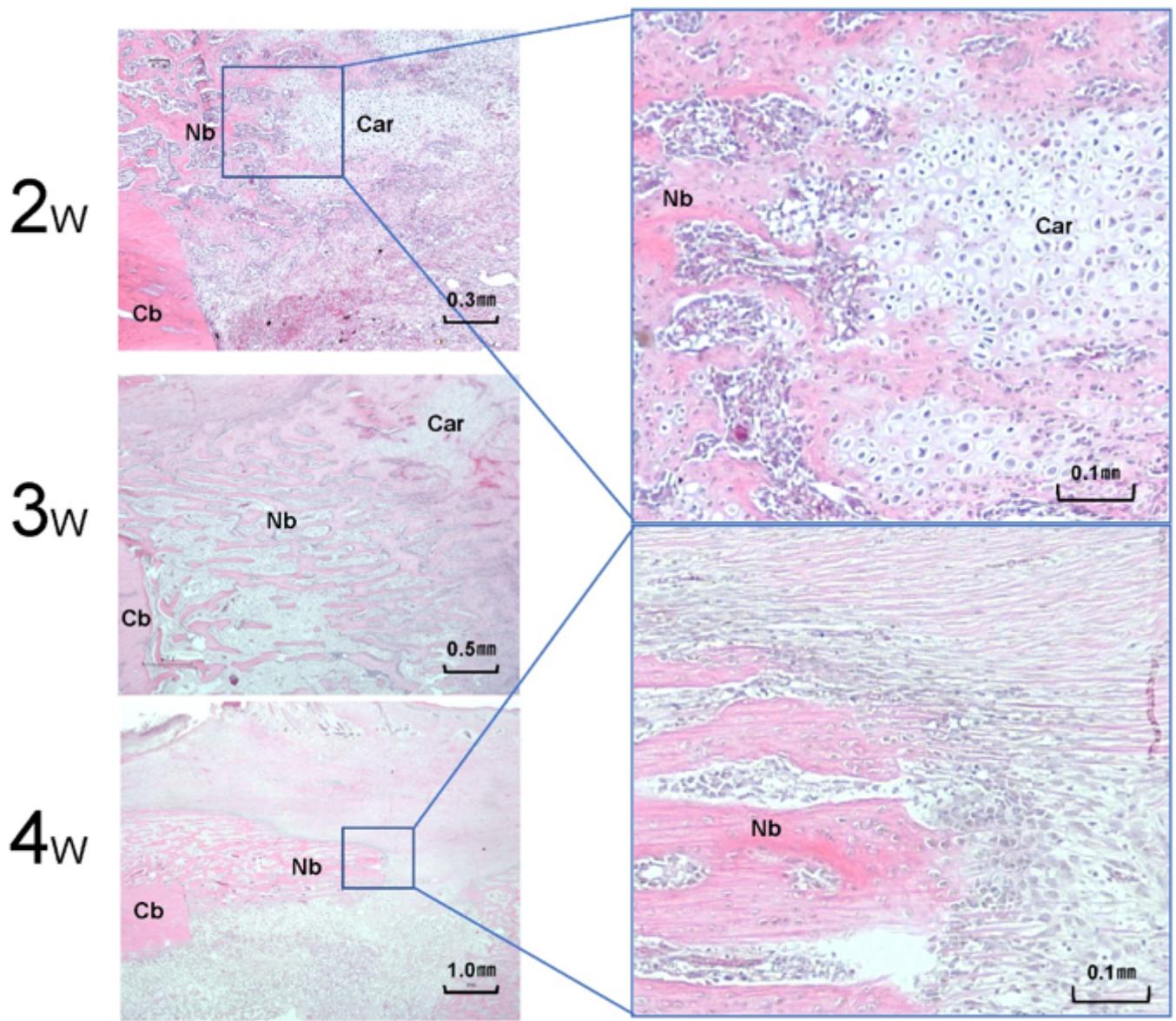


Figure 5

Histological analysis Representative histological sections at the distracted callus stained with hematoxylin and eosin. Chondrocytes were abundant in the distracted callus two weeks after the osteotomy, and a new bone was formed by endochondral ossification. The woven bone with some cartilage is predominant. Almost no chondrocytes were observed in the distracted callus four weeks after osteotomy, and a new bone was formed by intramembranous ossification. The mature lamellar bone is predominant. Car, cartilage cell; Nb, new bone; Cb, cortical bone