Locally administered single-dose teriparatide affects critical-size rabbit calvarial defects: A histological, histomorphometric and micro-CT study

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of teriparatide (PTH 1-34, rhPTH) on a rabbit defect model with local xenogen grafts histomorphometrically and radiologically.
Methods: For this purpose, two 10 mm diameter critical-size defects were created in the calvaria of 16 rabbits. In the control group, the defect area was filled with a xenogen graft, while in the teriparatide group (PTH 1-34), a xenogen graft combination with 20 mcg teriparatide was used. For both 4 − week and 8 − week study groups, new bone, residual graft, and soft tissue areas were evaluated as well as bone volume histomorphometrically and radiologically.
Results: Histomorphometrically, there was a significant difference in new bone area values at the 8th week ($p < 0.05$), but there was no significant difference between the 4 − week values ($p > 0.05$). There was no statistically significant difference between the groups at both 4 and 8 weeks ($p > 0.05$). In the radiologically measured total bone volume values, PTH1-34 group values were found to be significantly higher for both 4 − and 8 − weeks values compared to the control groups ($p < 0.05$).
Conclusion: In this study, rhPTH, which is used locally in defect areas to be repaired with bone grafts, increases both new bone volume and total bone volume.

Introduction

Bone grafting is a method commonly used to compensate for hard tissue loss in orthopaedic surgery. This method is frequently needed to repair bone defects due to tumor surgeries, bone atrophy, injuries, or congenital malformations.[1] There are a number of natural and synthetic graft materials that are used for this purpose. Because they contain osteogenic cells and osteoinductive factors that play key roles in bone regeneration, autogenous grafts have been considered the gold standard in bone

replacement for many years. The biggest disadvantages of autogenous bone grafts are the increase in morbidity, as they require a second surgical area, and the complications that may arise during or after surgery. Therefore, the use of other types of grafts, alone or in combination with other materials, has started to gain ground in clinical practice in recent years.[2]

Bone remodeling is a continuous process that consists of bone matrix synthesis and mineralization. The calcium metabolism required for this process is regulated by three hormones: parathyroid hormone (PTH), vitamin D (calcitriol), and calcitonin.[3,4] PTH is an endogenous protein that consists of 84 amino acids. It has direct and indirect effects, particularly on bone remodeling, the kidneys, and the intestines. It also acts continuously to preserve the optimal concentration of calcium ions in the blood and is used in the treatment of osteoporosis.[3,5] Teriparatide (PTH1-34, rhPTH) is a synthetic recombinant human protein that consists of the first 34

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amino acids of PTH[6] and has both anabolic and catabolic effects on bone. When administered systemically at intervals, it is a strong inducer of osteoblastic activity. Continuous treatment, on the other hand, increases osteoclast density and activity, which increases bone resorption. Thus, it negatively affects bone turnover.[7,8]

rhPTH is known to increase the differentiation of osteoprogenitor cells into osteoblasts, inhibit osteoblast apoptosis, and, as a result, cause increased bone formation. In addition, it is thought to suppress apoptosis by deactivating proteins required for apoptosis and increasing proteins required for cell survival. Moreover, it can stimulate insulin growth factor-1, which is known to increase cell proliferation and decrease apoptosis.[9] Furthermore, an increase in PTH can increase trabecular and cortical thickness, thus improving overall bone density and mass.[8,10]

There are two types of bone healing mechanisms: intramembranous ossification and endochondral ossification. In intramembranous ossification, MSCs differentiate to osteoblasts, creating bone tissue directly in an anabolic process (typical of flat bones). In endochondral ossification, mesenchymal stem cells differentiate into chondrocytes, which create cartilage tissue. This cartilage tissue is mineralized with extracellular matrix through chondrocyte apoptosis. Subsequently, the osteoblast cells penetrate this structure and lay down the bone tissue. Long bones typically grow and heal by this process.[11] However, there is no information that differentiates the effects of rhPTH on flat bones or long bones.

The aim of this study was to evaluate the effect of local administration of single-dose rhPTH with xenografts on bone healing in rabbit calvarial bone defects by means of micro-computed tomography (CT), as well as via histological and histomorphometric analyses.

Materials and methods

Experimental model

The study was conducted on 16 adult male New Zealand rabbits, each weighing about 3500 g. The subjects were kept in appropriate cages in a setting with 12 h of light, 12 h of darkness, and a temperature of $22 \pm 2\,^\circ$C. At least 1 week prior to the surgical operation, the subjects were transferred to the laboratory environment, where the experiments were to be conducted, to provide sufficient healthcare, protect the subjects against infections, allow them to adapt to their new environment, and evaluate their overall health. The subjects were fed standard laboratory feed. To provide easy access to water and food, allow sufficient room to move around, and create a less stressful environment, subjects were kept in separate cages.

Two groups were designed, with one half of each experimental animal's calvarium (right and left) in the following groups:

- Teriparatide + Xenograft group (PTH1-34)
- Xenograft/Control group (C)

Each group was divided into two healing period time frames, 4 and 8 weeks, for evaluation. In each experimental animal, the PTH1-34 group was located on the right calvarium, and the C group was located on the left calvarium.

Surgical procedure

For anesthesia, all experimental animals were administered 35 mg/kg of ketamine hydrochloride (Alfamine, Alfasan International BV, Woerden, the Netherlands) and 2.5 mg/kg of xylazine hydrochloride (Alfazyne, Alfasan International BV, Woerden, the Netherlands) intramuscularly. The left and right cranial areas were

shaved, and the surgical area was cleaned using povidone iodine (Batticon, Adeka, Istanbul, Turkey). To control bleeding, 1 ml of local anesthetic solution (Ultracain DS Forte, Sanofi Aventis, Istanbul, Turkey) was infiltrated into the relevant area. A full thickness incision, including the periosteum, of approximately 4 cm was made along the midline of the calvarium using a No. 15 surgical blade, and the bone surface was exposed. Two osteotomies were then performed on the parietal bone, to the left and right of the midline, using a trepan drill with an outer diameter of 10 mm and inner diameter of 9 mm, and cooled with sterile saline all without damaging the dura (Fig. 1). Since the 10 mm diameter was determined to critical size, it was similarly used in this study.[12,13]

For the PTH1-34 group, each defect was grafted with 0.2 cc of bovine graft with a particle size of 0.5–1 mm (BEGO Oss, BEGO Implant Systems GmbH & Co. KG, Bremen, Germany), and 20 µg of teriparatide (250 µg/ml teriparatide, Forsteo, Eli Lilly and Co., Indianapolis, IN, USA) was injected into the grafted area, which was then covered with a slow-rate resorption collagen membrane (Collagene AT; Sistema AT, Padova, Italy). For the C group, each defect was grafted with 0.2 cc of bovine graft with a particle size of 0.5–1 mm (BEGO Oss) and covered with a slow-resorption collagen membrane (Collagene AT).

Finally, the dermal and subdermal tissues were primarily closed with 16 mm of 4-0 resorbable polyglactin suture (Coated Vicryl, Ethicon, Johnson & Johnson, St. Stevens-Woluwe, Belgium) on a 3/8 needle. Wound dressing spray (Opsite, Smith & Nephew, Mississauga, Ontario, Canada) was applied to the suture area to prevent post-operative infection.

In the post-operative period, the subjects were administered, via intramuscular injection, 1 mg/kg of meloxicam (Maxicam X4, Sanovel, Istanbul, Turkey) as an analgesic and 2.5 mg/kg of enrofloxacin (Baytril-K 5%, Bayer, Kansas, USA) as an antibiotic, for 5 days. The subjects were kept in separate cages throughout the experiment with periods of 12 h of light and 12 h of darkness. The ambient temperature was 22–24 °C, and humidity was 55–70%. The wound areas were checked on a regular basis, and sufficient food and water were provided.

Tissue processing

Half of the subjects were euthanized at the end of the 4th week and the other half at the end of the 8th week, via an intramuscular injection of a lethal dose of xylazine hydrochloride (30 mg/kg) and ketamine hydrochloride (70 mg/kg). The defect areas were
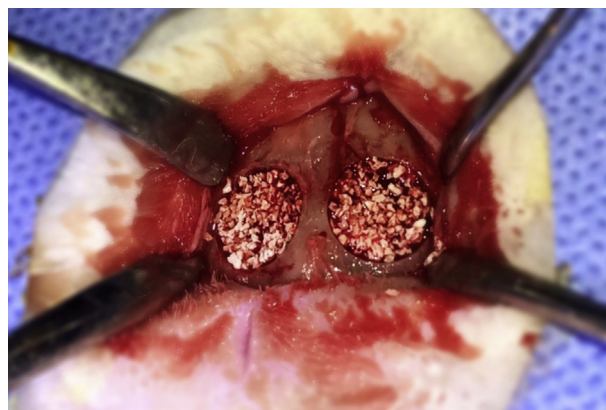

Fig. 1. Protocol for bone grafting of calvarial bone defects in a rabbit model. Two bone defects measuring 10 mm in diameter were created on each side of the calvaria. Xenogeny graft material was used to fill the defect on each side and the right one was treated with teriparatide. The grafted area was covered with a collagen membrane.

identified and removed *en bloc* from the cranium of each rabbit, along with some intact bone tissue. Then, samples were divided into groups for each rabbit and fixed for 48 h in 10% buffered formaldehyde.

Radiological analysis

The specimens were scanned with a micro-CT scanner (Skyscan 1174, Skyscan, Kontich, Belgium) with a pixel size of 40 μm. The x-ray tube voltage was 50 kV, the current was 800 μA, and the exposure time was 2300 ms. X-ray projections were obtained at 0.70° intervals with a scanning angular rotation of 180°. Raw data obtained in the scanning stage were reconstructed using the NRecon (NRecon version 1.6.9.4, Skyscan, Kontich, Belgium) software provided by the manufacturer. Eight-bit grayscale images, reconstructed using NRecon, were transferred to CTan (version 1.13.5.1, Skyscan, Kontich, Belgium) software. Values for basic indicators, such as bone volume, bone surface area, soft tissue volume, soft tissue surface area, bone/soft tissue ratio, and bone density, were obtained for the regions of interest selected.

Histomorphometric analysis

Histomorphometric analysis was performed by an examiner who was blinded to the identity of the samples. Samples were decalcified for 21 d in a 10% acetic acid solution that was replaced every 3 d. Tissues were then washed with distilled water and dehydrated by passing them through an alcohol series. Then, tissues were made transparent with a xylene series and blocked by embedding in paraffin wax. A series of transverse cross sections with a thickness of 4—6 μm was taken from all blocks, with a sampling ratio of 1/20, and placed on glass slides to assess the histological structure. The cross-sections were deparaffinized by xylene in a 60 °C incubator, dehydrated, and then stained with hematoxylin eosin (H&E). All stained cross-sections were covered with Canada balsam. Histomorphometric analyses were performed via a computer-assisted image analyzer programme (Leica Qwin plus V3, Leica-Germany). Five different pictures were taken of each cross-section at 100× magnification. The surface areas of new bone trabeculae and soft tissue filling the defect regions were calculated in μm^2 units.

Statistical analysis

The data were analyzed using the statistical software package SPSS 24.0 (SPSS Inc., Chicago, IL, USA). Measurements were reported as the mean ± standard deviation. In addition to descriptive statistics (mean, median, and standard deviation), the Mann Whitney U test was used to compare independent groups in the analyses. In each group, the comparisons of the 4-week and the 8-week data were analyzed by Wilcoxon test. p values that were smaller than 0.05 were considered statistically significant.

Results

An examination of the histological preparations of the 4-week samples showed that in all of them, the defect area consisted of interconnecting new bone trabeculae and loosely collagenized connective tissue. The connective tissue gained a cellular collagenized character as it approached the center of the defect. New bone generation was observed taking place in a centripetal manner, from the edges of the defect towards the center. This centripetal tendency was more salient in the PTH1-34 group. New bone trabeculae had formed around the graft particles, but most were localized in the periphery of the defect, where they took the form of

trabeculae due to the reactivation of the damaged periosteum. The histological appearances of the 8-week samples were similar, overall, to those of the 4-week samples from the same group, but there were more bone trabeculae. There were also fewer graft particles between the trabeculae compared to those in the 4-week samples. Furthermore, there was more bone generation toward the center, but bone formation did not fill the entire defect (Fig. 2).

Quantitative results of the histomorphometric analysis of the bone defects showed the new bone area at 8 weeks to be larger than that at 4 weeks in all groups. At 4 weeks, there were no significant differences between the two groups in terms of new bone area (p > 0.05). At 8 weeks, the PTH1-34 group had significantly more bone surface compared to that of the C group (p < 0.05; Fig. 3).

In terms of residual graft area, there were no significant differences between the groups, either at 4 weeks or 8 weeks (p > 0.05). When residual graft areas at 4 weeks and 8 weeks were compared within each group, no statistically significant difference was found (p > 0.05; Fig. 3).

Histomorphometric analysis of the soft tissue values showed that there were no significant differences between the two groups at 4 weeks (p > 0.05), but the PTH1-34 group had significantly lower values at 8 weeks compared to those of the C group (p < 0.05). There were statistically significant differences between the 4-week and 8-week values of the PTH1-34 group (p < 0.05), while the differences between the 4-week and 8-week values of the C group were not statistically significant (p > 0.05; Fig. 3).

The radiological examination showed that the total bone volume of the PTH1-34 group was significantly higher compared to that of the C group, both at 4 weeks and at 8 weeks (p < 0.05). In both groups, the 8-week values were significantly higher compared to those of the 4-week values (p < 0.05; Figs. 4 and 5). The bone density was not significantly different among the groups (p > 0.05).

Discussion

In recent years, systemic treatment by the administration of rhPTH at daily intervals has been shown to improve bone healing in calvarial defects in rats,[14,15] implants placed in the tibia,[4,16] periodontal defects,[17] and distraction osteogenesis in rabbits.[18,19] However, the effects of local administration of rhPTH have not been completely evaluated. This study began with the hypothesis that locally administered rhPTH with a xenograft would result in faster ossification by inducing osteoblastic activity, and the data yielded the expected result.

In similar animal studies, areas for introducing experimental defects were identified as the mandible, calvarium, femur, tibia, fibula, and radius. To create both groups in the same animal and anatomical region and prevent individual differences, the calvarium was selected as the surgical area in the present study. Although the critical-size aspect of this area is debated, many studies have shown less than 20% ossification in bilateral calvarial defects with diameters near 10 mm and recommended it as the critical size.[12,13] Following this recommendation, the present study used calvarial defects with diameters of 10 mm.

Previous studies on bone formation in rabbit calvarial defects used 4 weeks and 8 weeks as the healing periods. Sohn et al examined the spontaneous healing potential of critical-size defects in rabbit calvaria, and concluded that 2 or 4 weeks would be appropriate for observing the early effects of the healing period in this defect model, and 8 or 12 weeks to observe the late effects.[20] The present study used 4 and 8 weeks as the healing periods to evaluate both the early and late phases of bone formation, along with the resorption of the materials used.
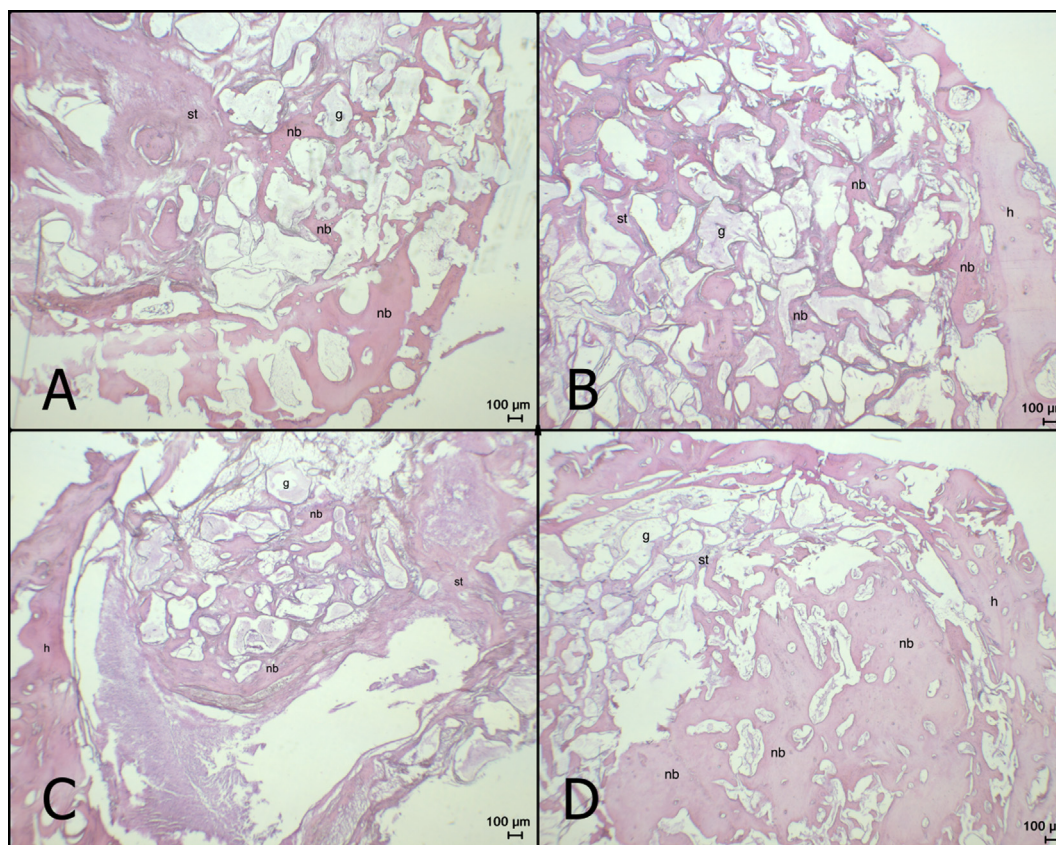
Fig. 2. Histological findings 4 and 8 weeks after surgery for calvarial bone defects in a rabbit model. A, at 4 weeks, specimens in the C group show that the defect area consists of anastomosing new bone trabeculae (less than in the PTH1-34 group) and loose collagen connective tissue (H&E, × 20). B, at 4 weeks, new bone trabeculae were observed towards the center of the defect at the continuation of the host bone trabeculae specimen in the PTH1-34 group. The loose collagen ligament in the periphery of the defect was noted to be in a central collagenous structure (H&E, × 20). C, specimens in the C group at 8 weeks showed anastomosing new bone trabeculae (less than in the PTH1-34 group), graft particles, and cellular structured collagen connective tissue (H&E, × 20). D, at 8 weeks, specimens in the PTH1-34 group showed formation of anastomosing new bone trabeculae, graft particles, and cellular structured collagen connective tissue (H&E, × 20). (H&E, hematoxylin and eosin; C group, xenogeny bone grafting only; PTH1-34 group, teriparatide with xenogeny bone grafting; nb, new bone trabeculae; g, graft material; st, soft tissue; h, host bone).

Autologous grafts used in the repair of bone defects can be obtained from different regions in the body. They stimulate new bone formation due to their osteogenic properties. Because of this feature, they are called the gold standard and the first choice as a bone graft material. However, since it requires a second operation area; increases surgical time, morbidity, postoperative discomfort, recovery time and costs. As a result, the use of other graft materials has become more common in clinical practice and one of them is bovine bone graft. In addition, the rate of resorption in autologous grafts is still a serious disadvantage.[21] Xenografts, such as bovine bone grafts, rescues the patient from autologous bone collection, provides a stable scaffold for bone formation and maintains graft volume stability over the long term due to low resorption rate.[22] Bezerra et al showed that; there was no statistically significant difference between the autologous grafts and xenografts administrated with platelet rich plasma (PRP) used in the reconstruction of alveolar clefts.[2] Because of these properties, bovine bone graft was preferred in this study.

Achieving faster ossification of bone grafts has become one of the most important goals in contemporary surgical treatment. Therefore, many studies have applied a large number of chemical and biological molecules, from statins to stem cells and from bone morphogenetic proteins to bisphosphonates, locally or systemically, and examined their interactions with the bone tissue. PTH is a polypeptide hormone consisting of 84 amino acids and exerts its biological effect via the 1-34 segment on the N-terminus region.

This functional segment, known as rhPTH, has been shown to increase total bone mass by accelerating bone formation.[18] Systemic administration of rhPTH at daily intervals on spongy bone is thought to improve and strengthen the bone microstructure; it has been shown to exert this effect by increasing the connection between cortical and spongy bone and changing the structure of the trabeculae from a rod-like to a plate-like form.[23] On the other hand, rhPTH was thought to increase cortical bone porosity and decrease cortical bone strength. However, studies conducted in recent years showed that rhPTH significantly increases cortical bone width and does not have a significant impact on porosity.[23–25] In addition, studies have shown that rhPTH increases the production of bone matrix proteins by stimulating earlier proliferation and differentiation of osteoprogenitor cells, thus resulting in increased cortical and spongy bone formation.[26–28]

Many animal studies from fracture healing models to implant osteointegration models have demonstrated the positive effect of systemic administration of rhPTH at intervals on bone healing.[18,29] However, there are very few studies on the effect of a single dose of rhPTH administered locally during surgery. One study conducted on rats examined the systemic effects of single dose local administration, in order to avoid multiple repeated administrations and to eliminate systemic effects; serum calcium concentrations immediately prior to euthanasia at 15 days and 60 days were compared, and no statistically significant differences were found. In this way, they showed that locally administrated rhPTH increased
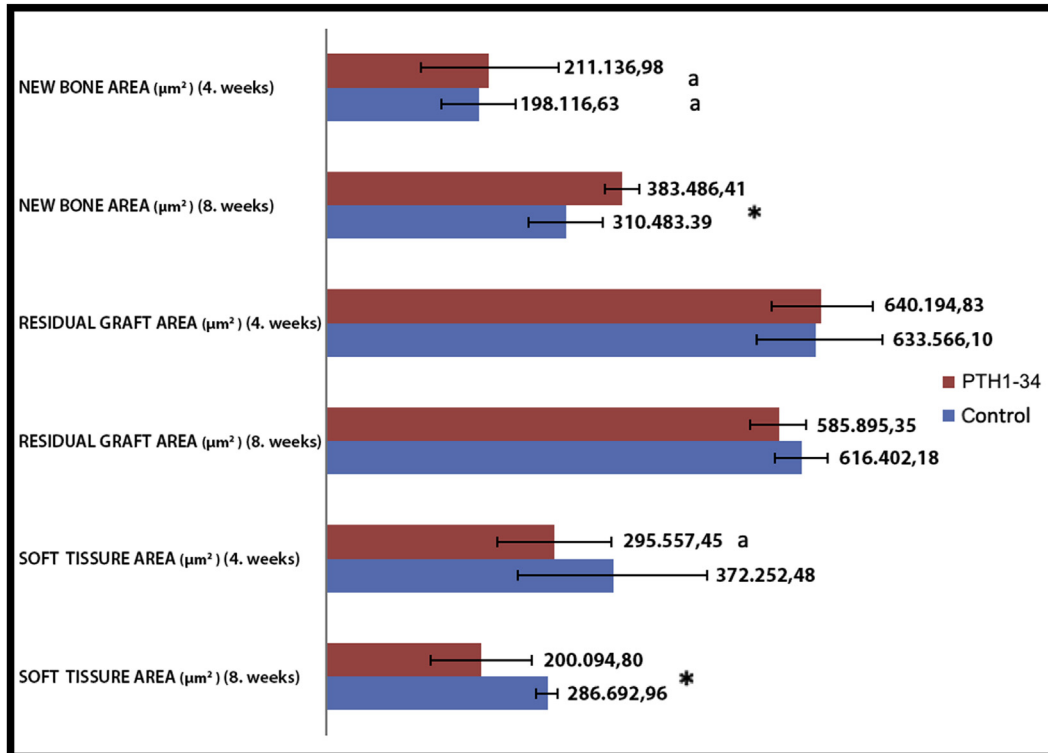
Fig. 3. New bone, residual graft and soft tissue area measured by histomorphometry at 4 and 8 weeks after surgery for calvarial bone defects in a rabbit model. Data are presented as the mean ± standard deviation. (C group, xenogeny bone grafting only; PTH1-34 group, teriparatide with xenogeny bone grafting; a, significantly different from values obtained 8 weeks after surgery; *, significantly different from values obtained in the other group, black line indicate the standard deviation).

Fig. 4. Comparison of values for the total bone volume derived from radiographs obtained 4 and 8 weeks after surgery. Data are presented as the mean ± standard deviation. (C group, xenogeny bone grafting only; PTH1-34 group, teriparatide with xenogeny bone grafting; a, significantly different from values obtained 8 weeks after surgery; *, significantly different from values obtained in the other group, black line indicate the standard deviation).
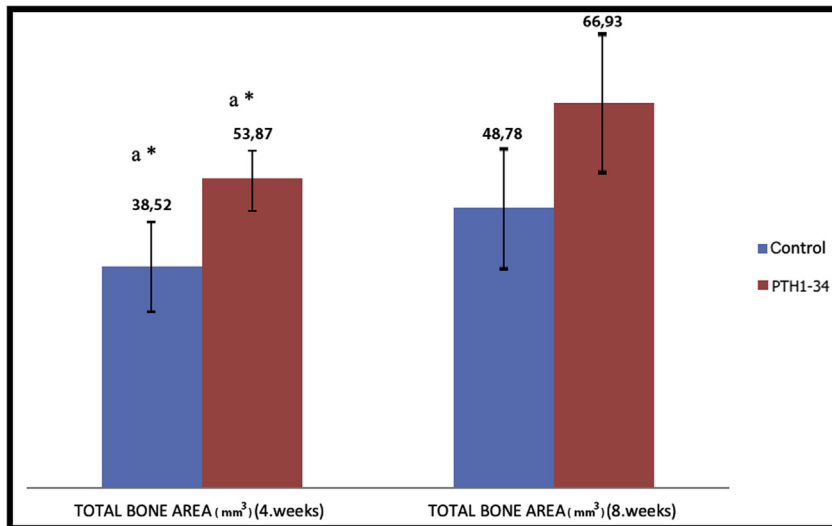
ossification without systemic findings such as serum calcium concentration.[8] The present study has shown that local administration of rhPTH positively impacts bone healing.

Auersvald et al examined the effect of 20 μg of rhPTH on the healing of defects in rat calvaria using micro-CT, along with histological and histomorphometric evaluation, and they showed that rhPTH has a positive effect on areas of new bone.[8] Although there are no studies in the literature on the optimal dose, the present study used a dose of 20 μg for the PTH1-34 group and found statistically significant and positive differences. Data from future studies regarding dosages will likely allow for even better results.

Although there have been a few studies on the local administration of rhPTH, the carrier systems used to deliver rhPTH to the defect area have not been thoroughly evaluated. Some studies used a collagen sponge as the carrier, whereas others chose to drip the material on the relevant area.[8,30] The present study also used the
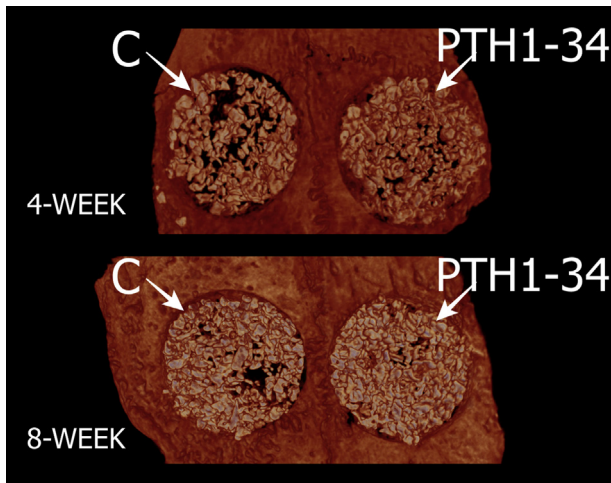
Fig. 5. Micro-computed tomography (CT) images of all groups at 4 weeks and 8 weeks.

method of dripping the rhPTH on the grafted defect in the PTH1-34 group, thus using the bone graft as the carrier. Therefore, equal amounts of rhPTH and bone graft were applied to each defect. Use of other carrier systems, such as membranes and platelet-rich fibrin membranes, to deliver rhPTH in future studies would help to identify the most appropriate carrier system for local administration of rhPTH.

The skeletal system contains bones that are inherently loaded by distinct mechanical force patterns. Long bones are loaded predominantly along the longitudinal direction with much higher amplitude than flat bones such as calvaria that are loaded radially and tangentially by intracranial pressure and mastication. As a sample, the human fibula is estimated to have a load that is nearly twice that of the skull bone. Extracellular mechanical forces are coupled to the intracellular organization of the cytoskeleton that regulates cellular connections to other cells and the extracellular matrix, thus affecting cell shape and functional outputs. So, the morphology of osteocytes, the patterning of the osteocyte network and their function are determined by external loading whose directional patterns and amplitudes are physiologically different in each bone.[31] This study is valuable for showing the effect of rhPTH on healing in flat bones. However, further studies are needed to compare this effect with the effects of rhPTH on long bones.

The most reliable methods for measuring the amount of new bone and soft tissue formation are two-dimensional histomorphometric analysis and three-dimensional micro-CT analysis. However, in histomorphometric analysis, wider segments are examined compared to those of micro-CT analysis. Therefore, studies conducted with micro-CT analysis are reported to provide more accurate and detailed results.[32] Additionally, samples examined using micro-CT do not deteriorate, thus allowing histomorphometric analysis to be made. In the present case, histomorphometric results were supported with radiological results.

Conclusions

This study showed that rhPTH locally administered to a bilateral rabbit calvarial defect model increased both new bone volume and total bone volume. In addition, this was the first study in which local administration of rhPTH was used in conjunction with xenografts; notably, there have been a few studies regarding local application of rhPTH, such that the positive effects of rhPTH have been shown in the context of different graft materials. And this

study showed that, xenografts are a good carrier system for rhPTH. However, further studies are needed with different doses, graft materials, and carrier systems using different animal models.

Ethical approval

This study was approved by decision 2017/53-05 of Hacettepe University's Local Ethics Committee for Animal Experiments, dated September 26, 2017. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Conflicts of interest

All authors have no conflicts of interest.

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