Enhancement of Bone Augmentation in Osteoporotic Conditions by the Intermittent Parathyroid Hormone: An Animal Study in the Calvarium of Ovariectomized Rat

Tatsuya Kubota, DDS¹/Akira Hasuike, DDS, PhD²/Masako Naito, DDS, PhD³/Katsuyoshi Tsunori, DDS, PhD⁴/Seiko Min, DDS, PhD⁵/Shuichi Sato, DDS, PhD⁶

Purpose: Intermittent parathyroid hormone (PTH) is the commonly used therapeutic approach for patients with severe osteoporosis. The goal of this study was to elucidate the effect of the intermittent PTH treatment on guided bone augmentation (GBA) in the calvarium of ovariectomized (OVX) rats. Materials and Methods: Surgical ovariectomy on 14 rats and sham surgery on 7 rats were conducted on all rats as the first surgery. GBA surgery was conducted 8 weeks following the first surgery in the rat calvarium by placing 5-mm-diameter cylindrical plastic caps. Following surgery, rats were treated with 40 μg/kg PTH (OVX-PTH) or saline (Sham-Saline, OVX-Saline) via intraperitoneal injection three times per week during the all-observational period. Longitudinal microcomputed tomography (micro-CT) imaging was performed every 2 weeks following the GBA surgery without euthanasia, and the amount of newly generated bone volume (BV) was calculated. All rats were euthanized 12 weeks after GBA surgery, and histology was obtained. Sections stained with hematoxylin and eosin were used for the quantitative analysis of newly generated tissue, and immunohistology was used to visualize Runx2-positive cells and TRAP-positive cells. Results: Throughout the monitoring period, the BVs of OVX rats without PTH treatment (OVX-Saline) were significantly lower than that of the other two groups at weeks 8 and 12 in micro-CT analysis. During all experimental periods, the BV was highest in the OVX rats that were treated with PTH (OVX-PTH). Histologic analysis confirmed the result of micro-CT, and determined that the OVX-PTH presented a greater number of Runx2-positive cells. The number of TRAP-positive multinucleated osteoclasts was highest in OVX-PTH rats; there were no significant differences between the other two groups. Conclusion: The results of this study suggest that treatment with intermittent PTH was associated with increased newly regenerated bone volume in ovariectomized rat calvarial bone augmentation, which may have important clinical implications. INT J ORAL MAXILLOFAC IMPLANTS 2018;33:1003–1010. doi: 10.11607/jomi.6326

Keywords: bone augmentation, GBR, osteoporosis, systemic disease
vessels to revascularize. Extensive patient-to-patient variability or site-related factors can sometimes affect the predictability of this treatment. Standardized animal models are essential tools in translational research and medical technology development, which can help overcome these challenges. The present group developed a guided bone augmentation (GBA) model using plastic caps placed on rat calvarium. Using this rat model, the effects of bone substitutes, hormones, and smoking on the quantity of newly augmented bone confirmed using microcomputed tomography (micro-CT) and histologic examination was previously documented.

Osteoporosis, which is defined as a systemic disorder, is characterized by low bone mass and architectural deterioration of bone tissue that makes bone more prone to fracture. Previous studies suggested that the bone regeneration and healing potential of the jawbone supporting the teeth decrease during osteoporosis. Hence, osteoporosis could be an inhibiting factor for osseous augmentation. The most common therapeutic options for osteoporosis are antiresorptive drugs, which inhibit osteoclast activity. Parathyroid hormone (PTH) is another option that is subcutaneously administered in post-menopausal women and men with severe osteoporosis, who are at a high risk for bone fractures. This treatment was developed by focusing on the anabolic effect in which PTH paradoxically caused bone formation when administered intermittently.

Both preclinical and clinical studies previously demonstrated that intermittent subcutaneous administration of PTH fosters osseous healing or regeneration. In the present authors’ previous study, the bone regenerative capacity of the intermittent PTH treatment was demonstrated in a GBA model using healthy rats. However, it is still unclear whether intermittent PTH administration has an effect in an osteoporotic condition that is similar to the healthy condition. This question is highly clinically relevant, as osteoporosis is common in elderly men and postmenopausal women, which is also the age group associated with the highest demand for dental implants.

Thus, the goal of the present study was to elucidate the effect of intermittent PTH treatment on bone augmentation in a rat model of osteoporosis. The administration of an ovariectomy (OVX) is known to be the most popular method of generating an animal model of estrogen deficiency, which is shown to be a good first predictor of treatment potential for osteoporosis. Thus, the rat calvarial GBA model was used in OVX rats in the present study. Dynamics of bone augmentation were examined through assessing micro-CT images, histology, and immunohistochemistry.

MATERIALS AND METHODS

Animals
A total of 21 6-week-old female Wistar rats, weighing 120 to 140 g each, were purchased from Nihon Clea for this study. The rats were maintained in plastic cages in a temperature-controlled room (22°C) with 55% humidity, and cycles of 12 hours of light followed by 12 hours of darkness. Rats were fed a normal pellet diet and tap water. The Animal Experimentation Committee at Nihon University (AP15D007) approved this study.

Surgical Procedure
The present study consisted of one experimental group (OVX-PTH) and two control groups (ie, OVX-Saline and Sham-Saline). The OVX or sham procedure was performed in the 6-week-old rats prior to the GBA surgery. Ovariectomy was performed in 14 rats. Following the administration of anesthesia, the ovaries were exposed by cutting through the muscle layer under a midline dorsal skin incision, and both ovaries were removed after pinching the ovary ducts, including neighboring subcutaneous fat with tweezers. The incision was sealed with 5-0 silk sutures. The sham group underwent the same anesthetic, incision, and suture procedures, but only neighboring subcutaneous fat tissue equal in size to the ovaries was ligated and removed.

The bone augmentation procedure was performed 8 weeks after the first surgical procedure. As previously reported and standardized by the present group, GBA was modeled in the rats by positioning plastic caps on the rat calvarium. After premedication with inhalation of isoflurane, the experimental rats were anesthetized with intraperitoneal butorphanol tartrate (2.5 mg/kg), midazolam (2.0 mg/kg), and dexmedetomidine hydrochloride (0.15 mg/kg). The local administration of 500 μL of a 1:80,000 dilution of lidocaine (Xylocaine, Astra Zeneca) was added for anesthesia and hemostasis of the surgical field. The dorsal part of the cranium was shaved and disinfected for surgery. A cutaneous flap was created by making a 40-mm skin incision along the sagittal suture and reflecting it laterally from the dorsal part. Circles with diameters of 5 mm were engraved on both sides of the calvarium by drilling a trephine. Five small penetrations were made by drilling a small round bur to induce bleeding from the bone marrow (Fig 1a). All drilling procedures were carried out under profuse sterile saline irrigation, and extreme care was exercised not to penetrate the dura. A cylindrical plastic cap was pressed into the circular notch and fixed with 4-META resin (Fig 1b). In all animals, the skin was subsequently carefully sutured with resorbable 4/0 polyglactin sutures.
PTH Treatment in OVX-PTH Rats
Following surgery, seven OVX rats were treated with intraperitoneal injections of 40 μg/kg synthetic human PTH (1-34 PTH, Asahi Kasei Pharma), three times a week, during the observational period (OVX-PTH). The concentration of PTH was adjusted with solution of 0.1 M Tris-HCl, at pH 7.5, containing 2% bovine serum albumin. Additionally, seven OVX rats (OVX-Saline) and seven sham rats (Sham-Saline) were injected with saline as a control.

Body Weight Monitoring
All rats were weighed weekly, beginning on the day of the OVX or sham surgery.

Micro-CT Image Analysis
A total of 21 rats and 42 sites were evaluated. Repeated micro-CT imaging was performed using a micro-CT system (R_mCT2 system) without euthanasia. The rat calvarium was scanned using micro-CT with an x-ray source of 90 kV/100 A, at 0, 2, 4, 6, 8, 10, and 12 weeks after GBA surgery. After anesthesia with inhalation of isoflurane, each rat was placed on the stage. The micro-CT imaging range was defined as regions of interests (ROIs) that had an area of 5.0 \( \times \) 3.0 mm\(^2\) (Fig 1c). The original three-dimensional (3D) images were displayed using i-View software (Kitasenju Radist Dental Clinic, i-View Image Center). The bone volume (BV) of each ROI was measured from the number of bone-associated voxels using measurement software (Kitasenju Radist Dental Clinic, i-View Image Center), and calculated by subtracting bone volume on day 0 from each subsequent bone volume.

Histologic Analysis
The rats were euthanized 12 weeks after GBA surgery through CO\(_2\) asphyxiation, and their calvarium was removed and fixed in 10% formalin. The specimens were demineralized with formic acid-sodium citrate solution for 1 week, dehydrated, and embedded in paraffin blocks. The blocks were processed into semi-serial sections that were 4- to 5-μm thick. The sections were subsequently stained with hematoxylin and eosin (H&E). The total area of newly augmented tissue (TV) and newly generated bone (BV) was measured using ImageJ software (National Institutes of Health). Quantitative data were presented in two ways. The volume of newly generated bone was presented as mean values of percentage of BV within each plastic cap. The bone density was presented as a percentage of BV within the TV.

Further histologic analysis was conducted for the detection of osteoblasts and osteoclasts. Sections were deparaffinized, and immersed in 3% H\(_2\)O\(_2\) in methanol for 15 minutes at room temperature to quench endogenous peroxidase. For the detection of osteoblasts, the slides were incubated with human anti-Runx2 antibodies (MBL), for 1 hour at room temperature according to the manufacturer’s protocol. The specific reaction for the immune complex was detected using 3’3’-diaminobenzidine tetrachloride (DAB; Merck). Serial sections were also stained for tartrate-resistant acid phosphatase (TRAP), a marker of osteoclasts and osteoclast-like cells, as described previously.\(^{11}\) ROIs were virtually set in the center of each section, and Runx2-positive cells and TRAP-positive multinucleated cells were counted in the regions. The sizes of regions were different between the two types of cells, ie, 800 \( \times \) 800 μm\(^2\) for Runx2-positive cells and 4,000 \( \times \) 1,000 μm\(^2\) for TRAP-positive cells. The numbers of each type of cell were recorded as the mean cell number per 10\(^6\) μm\(^2\).

Statistical Analysis
A one-way analysis of variance (ANOVA) was used to analyze the BV, percentage of area newly generated within each cap, and numbers of Runx2/TRAP-positive cells. Pairwise comparisons were performed using the Tukey post hoc test when significant differences were found. All statistical analyses were conducted using GraphPad Prism 5 for Windows software (GraphPad Software). The level of significance was set at a P value of .05.

RESULTS
All surgeries were uneventful. No inflammatory response was observed at any of the surgical sites.
Measurement of Body Weight

The body weights were recorded and are summarized in Fig 2. There were no significant differences in initial body weights among the three animal groups. All animals presented a sharp increase in body weight by the seventh week after the first surgery (ovariectomy), and a gentle increase from the eighth week to the end of the monitoring period. Differences in body weight were not confirmed between the OVX-PTH and OVX-Saline rats throughout the experimental period. Both OVX group rats had higher body weights than Sham rats, and differences in body weight were maintained until the end of the observations ($P < .05$).

Micro-CT Imaging Analysis

Hyperdense contrasts were scarcely seen in all rats at 0 and 4 weeks (Fig 3). Hyperdense images creeping up along the internal side of the plastic caps were confirmed at weeks 8 and 12 in all three groups. In the OVX-PTH and control groups, hyperdense layers occupied the bottom half of the caps at week 12. Nevertheless, the OVX-Saline group demonstrated reconstruction at the same height as the other two groups, and hypodense areas were confirmed just above the bottom of the ROIs at weeks 8 and 12.

Quantitatively, the BV of the micro-CT images gradually increased over time in all groups from 0 to 12 weeks (Fig 4). Significant differences between the three groups were observed at weeks 8 and 12. The BV of the OVX-Saline group was lower than that of the other two groups at weeks 8 and 12. In the OVX-PTH group, the BV increased between weeks 4 and 8. Consequently, the BVs of OVX-PTH were higher compared with the other two groups at weeks 8 and 12. During all evaluation phases, the BV of the OVX-PTH group was the highest among the three groups.

Histomorphometry and Immunohistologic Analyses

Figure 5 shows the histologic sections (stained with H&E) of the augmented bone at 12 weeks. Thin and sparse trabecular bone patterns, with the absence of
Bone volumes (BV) of each experimental group. The BV was calculated as the number of voxels multiplied by the voxel volume. *Vs OVX-Saline at $P < .05$; #Vs the other two groups at $P < .05$.

Histologic sections at week 12 representative of each group. (a to c) OVX-PTH. (d to f) OVX-Saline. (g to i) Sham-Saline. Sections (a, d, g) stained with hematoxylin and eosin and (b, e, h) immunostained with anti-Runx2, and (c, f, i) stained with tartrate-resistant acid phosphatase (TRAP).
connectivity and presence of abundant fibrous connective tissues, were confirmed in OVX-Saline group rats. Quantitatively, only approximately 12% of bone fill was present in the OVX-Saline group, which was significantly lower than the other two groups (Fig 6a). Further, newly generated bone creeping up along the inner side of the plastic cap was observed in the PTH-OVX and Sham-Saline groups. In contrast to the Sham-Saline rats (23.6%), the percentage of ossified area was the highest in the OVX-PTH group (nearly 50%) (Fig 6a). PTH treatment significantly activated ossification in the augmented area not only in comparison with untreated rats, but also with healthy rats. The bone density (BV/TV) results showed similar tendencies as the newly generated bone volume (Fig 6b).

The presence of many osteoblast-like cells, identified using anti-Runx2 immunostaining, was confirmed in the plastic cap areas in the OVX-PTH rats. In contrast, only very few Runx2-positive cells were detected in the OVX-Saline rats in the area of the plastic cap (Fig 6c). In the OVX-PTH rats, Runx2-positive osteoblast-like cells were seen, not only in the area adjacent to the preexisting bone, but also in the newly generated tissue that was remote from the preexisting bone. The number of TRAP-positive multinucleated osteoclasts was much lower than Runx2-positive osteoblasts in all rats. The number of TRAP-positive multinucleated osteoclasts was highest in OVX-PTH rats; there were no significant differences between the other two groups (Fig 6d).

**DISCUSSION**

The present study demonstrated that OVX inhibits the bone regenerative ability of GBA. When OVX rats were treated with PTH, the enhancement of bone regeneration exceeded that of the control groups as confirmed using micro-CT analysis and histologic analysis. The imaging system used in the present study enabled investigation of bone structures without the need for euthanasia. Scanning was conducted every 2 weeks, with each rat being scanned a total of seven times. Miyahara et al.\textsuperscript{12} examined the biologic radiation effects of CT scanning with an identical system to the one used in the present study. They found no significant radiation effects, such as weight reduction, blood, and histopathologic disorder. Another group examined the skeletal parameters and bone marrow cells in the tibia of rats after eight weekly scans.\textsuperscript{13} The micro-CT system generated more than 20 times the radiation dose compared with the system used in the present study. They also reported no significant changes in skeletal and...
cell-related parameters. The results of these two studies, thus, indicated that micro-CT radiation has no effect on systemic and bone-related parameters.

Prior to GBA surgery, the gonads of rats in the experimental group were removed surgically to induce the osteoporotic condition. This is a well-proven animal model for osteoporosis studies, which is well-known to induce osteopenia and hyperphagia. As estrogen regulates food intake, the weight gain could be used to verify the reliability of the OVX procedure.14 The data presented in Fig 2 demonstrate the marked changes in body weight in the OVX rats and indicate that the ovariectomies were performed successfully in the present experiment.

Previous preclinical and clinical studies indicated that osteoporosis could be a potential risk factor for failures of bone regeneration,15 osseointegration,16 mandibular augmentation,17 and healing of dental post-extraction sockets.18 In the present study, histologic analysis of OVX-Saline rats demonstrates that osteoporotic conditions result in reduced trabecular volume and larger marrow spaces (Fig 6b). These results are in accordance with previous studies reporting a significant decrease in bone mineral density, changes in trabecular microarchitecture, and a decrease in mandibular cortical thickness.19,20 It may be inferred from histologic observations that the OVX procedure resulted in reducing the number of osteoblasts and increasing the number of osteoclasts. In the present study, the significant increase in the number of osteoclast-like cells was not confirmed. The number of osteoblast-like cells, however, was significantly lower in OVX-Saline rats compared with Sham-Saline rats. This result could be attributed to the lower volume of existing bone and fewer osteoclasts.

The effect of anti-osteoporotic medication (eg, bisphosphonates, PTH) on bone augmentation is also worth considering. There are conflicting issues about these effects, with some studies reporting that bisphosphonates may have a positive effect on bone regeneration following distraction osteogenesis21 and osseointegration,22 whereas others have documented that they failed to promote osseointegration of titanium domes with newly regenerated bone in rabbit calvarium.23 PTH has dual effects on bone metabolism, ie, whether it is anabolic or catabolic, depending on intermittent or continuous treatment, respectively. The anabolic effect is derived from reactivation of bone-lining cells, which are thought to be inactive osteoblasts; the initiation of osteoblastic bone formation occurs without osteoclastic bone resorption.24 It was also suggested that PTH administration delays the apoptosis of osteoblasts, hence prolonging osteoblast life.25 In previous studies, the present group examined the effects of intermittent PTH treatment in non-osteoporotic healthy rats using the calvarial defect model26 and GBA model.5 The histomorphometric and histologic analyses demonstrated that the amount of bone augmentation was increased significantly in PTH-treated healthy rats compared with the control rats at 12 weeks. The question remained as to whether intermittent PTH treatment influences the regeneration potential of GBR in osteoporotic rats. In the present study, higher BV values, as well as higher percentages of ossified areas and a higher number of osteoblasts, were demonstrated in the OVX-PTH rats using micro-CT analysis. Thus, it was clear that the anabolic effect of intermittent PTH treatment enhanced the osteogenic ability in the rat OVX model. Surprisingly, the enhancement of bone regeneration in OVX-PTH rats exceeded that of healthy rats (Sham-Saline), as confirmed using micro-CT analysis and tissue sections, ie, nearly 50% vs 35% fill, respectively. These data were beyond the scope of the authors’ expectations. The underlying mechanism of these results, however, remains unknown and requires further research.

The authors acknowledge the limitation regarding the examination of optimal PTH administration in the present study. In previous studies, the dosages of PTH for the anabolic effect varied depending on experiments, which ranged from 10 to 80 μg/kg.21,27–30 In the present authors’ previous study with healthy rats, high and low PTH dosage were compared, and it was found that a greater amount of bone augmentation could be achieved through a higher dose of PTH.5 Another group confirmed the anabolic effects of low-dosage PTH therapy on alveolar bone.28 Further, there is research examining the effect of topical administration.21 Considering the potential side effects, such as osteosarcoma and low blood pressure, it is important to pursue an effective and safe method of PTH administration. Thus, further studies are warranted to elucidate the optimal condition of PTH administration in this OVX rat GBA model.

CONCLUSIONS

The results of this study suggest that treatment with intermittent PTH was associated with increased newly regenerated bone volume in ovariectomized rat calvarial bone augmentation. This finding may have important clinical implications.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI grants JP16K20545 (Grant-in-Aid for Young Scientist [B]) and JP16K21408 (Grant-in-Aid for Young Scientist [B]). The authors would like to thank Asahi Kasei Pharma, Tokyo, Japan, for providing the PTH (1-34). The authors declare no conflicts of interest.
REFERENCES


27. Wronska TJ, Yen CF, Qi H, Dann LM. Parathyroid hormone is more effective than estrogen or bisphosphonates for restoration of lost bone mass in ovariectomized rats. Endocrinology 1993;132:823–831.


