



Original Article

Intermittent administration of parathyroid hormone improves bone quality and quantity around implants in rat tibiae

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ABSTRACT

Objectives: Intermittent injection of parathyroid hormone (PTH) is used to treat osteoporosis. The concept of bone quality was updated 20 years ago; however, these updates have not been adopted in implant dentistry. This study aimed to investigate the effects of intermittent administration of PTH on bone quality around implants in rat tibiae.

Methods: Grade IV-titanium-threaded implants that were 3.5 mm long and 2.0 mm wide were placed in a randomly selected side of the proximal tibiae of 12-week-old female Wistar rats. Three weeks after implant placement, the rats were randomly divided into PTH-administration and saline-injection groups (PTH and VC, respectively; n = 7 per group). Micro-computed tomographical, histomorphometric, and immunohistochemical analyses were performed to evaluate bone quality and quantity surrounding the implants.

Results: PTH significantly increased bone volume and bone mineral density in bones not associated with the implants as compared to these values in the VC group. PTH significantly increased bone area and the amount of collagen within the total inside areas of all implant threads compared to that observed in VC. Moreover, PTH significantly increased the number of osteoblasts, osteocytes, and osteoclasts in the total inside and/or outside areas of all implant threads and altered the ratio of type I and III collagen to total collagen fibers.

Conclusions: Within the limitations of this study, intermittent administration of PTH improved both bone quantity and bone quality based on the types and numbers of bone cells and the types of collagen fibers surrounding implants placed into rat tibiae.

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1. Introduction

Implant therapy is a major treatment option for missing teeth. Osseointegration surrounding dental implants is an important factor for determining the long-term clinical outcome of an implant [1]. Additionally, both bone volume and bone mineral density (BMD) in the jawbone are contributing factors [2,3]. In implant dentistry, BMD plays an important role in determining bone strength relative to bone volume, and it is believed to be synonymous with bone quality [4]. Bone quality is conventionally evaluated by bone toughness during the preparation of implant beds by drilling alveolar bone. However, in 2000, the National Institutes of

Health (NIH) proposed the new evaluation parameter “bone quality” [5]. According to this proposal, bone quality is completely independent of BMD. Bone quality is defined as “the sum of all characteristics of bone that influence the bone's resistance to fracture” [6] and refers to bone structures, bone turnover, mineralization, and damage accumulation [5]. We previously demonstrated that these components of bone quality are strongly correlated with bone cells, collagen fibers, and biological apatite under mechanical-loaded conditions [7–10].

Parathyroid hormone (PTH), which is produced by the parathyroid gland, controls serum calcium levels through its influences on bone tissue, the kidneys, and the small intestine. Animal and clinical studies clearly reveal that intermittent subcutaneous administration of PTH stimulates bone formation and increases the BMD of long and vertebral bones [11]. PTH is thus used for osteoporosis treatments that are approved by the Food and Drug

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Administration and other organizations. Intermittent administration of PTH stimulates new bone formation in rodent cancellous bones by inhibiting osteoblast apoptosis and reducing *Sost* expression in osteocytes [12–14]. The results from several animal studies have indicated that intermittent PTH injection enhances osseointegration and/or increases bone volume around implants placed into long bones [15–17]. Despite this, there is only sparse scientific evidence regarding the effects of intermittent PTH administration on bone quality around implants, as the updated concept of bone quality supported by bone cells, collagen, and biological apatite is not pervasive in implant dentistry. We hypothesize that intermittent PTH injection enhances both bone quantity and bone quality in the bone surrounding implants. The aim of this animal study was to investigate the effects of intermittent subcutaneous administration of PTH on bone quality around implants in rat tibiae.

2. Materials and methods

2.1. Animals, implant placement, and administration drugs

Female Wistar rats were used for experimental studies (12-weeks old, $n = 14$; Kyudo Co., Ltd, Saga, Japan). Grade IV-titanium-threaded implants that were 3.5 mm long and 2.0 mm wide and designed for rats had a thread pitch and height of 400 μm and 200 μm , respectively [10] ($n = 14$; Kyocera Co. Ltd., Kyoto, Japan). The rats were treated with systemic anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine), and an approximately 10 mm incision line was then prepared along the longitudinal axis of the tibiae. Skin flaps and the periosteum were elevated, and the implant was placed with its center at 7 mm from the end of a randomly selected side of the proximal tibiae. The skin was then sutured with 6–0 nylon (Natsume Seisakusho Co. Ltd., Tokyo, Japan). All rats were randomly divided into 2 groups 3 weeks post-implant placement, where one group intermittently received subcutaneous administrations of human PTH (pTH [1–34], BACHEM, Bubendorf, Switzerland) every 2 days (80 $\mu\text{g}/\text{kg}$ [18]) (PTH; $n = 7$), and the other group received saline injections (VC; $n = 7$) (Fig. 1A and B). Tibiae with associated implants were dissected at 5 weeks after the start of PTH administration. All animal experiments were performed according to the ARRIVE Guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>).

2.2. Microcomputed tomography (microCT)

Dissected tibiae were fixed in 10% neutral buffered formalin for 48 h at 4 °C ($n = 7$ each/group). As previously described, microCT scans were conducted at 20- μm voxel resolution with an energy level of 90 kV (R_mCT2; Rigaku Co., Tokyo, Japan) [19]. The regions of interest (ROIs) were determined between 5.0 mm and 5.5 mm from the proximal tibiae along the longitudinal axis to avoid influence from the implants. To confirm PTH effects on long bone, trabecular bone volume per tissue volume (Tb.BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular BMD (Tb.BMD), cortical bone ratio (cortical bone volume per total bone volume: Cv/Av), thickness of cortical bone (Ct.Th), and inner and outer perimeter of cortical bone (Ct.In.length and Ct.Ex.length, respectively) were semi-automatically measured using a TRI/3D-Bon (Ratoc System Engineering, Tokyo, Japan) according to published guidelines for microCT analyses [20].

2.3. Histology and immunohistochemistry

The dissected tibiae were fixed in 10% neutral buffered formalin for 48 h at 4 °C and then demineralized in ethylenediaminetetraacetic

acid (pH = 7.3, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) at 4 °C for 28 days ($n = 7$ each/group). The implants were then carefully removed with inverse rotation, paraffin-embedded, and cut into 5- μm -thick sections. The sections were stained for histomorphometric analyses incorporating hematoxylin and eosin (H&E), tartrate-resistant acid phosphatase (TRAP) (386 A; Sigma–Aldrich, St. Louis, MO), and picosirius red staining (Direct Red 80; Sigma–Aldrich) according to each manufacturer's protocol, and the sections were then visualized and photomicrographed under a light microscopy (Axio Scope A1; Zeiss, Oberkochen, Germany). Polarized light microscopy (Axio Lab. A1; Zeiss) was also used to detect picosirius red-stained type I and III collagen.

Bone area and osteocytes surrounding the implants were both quantitatively evaluated by H&E staining. Osteoclasts and collagen fibers were quantitatively assessed by TRAP and picosirius red staining, respectively. Histomorphometric analyses were performed using Zen2 software (Zeiss) and ImageJ (version 1.49; NIH, Bethesda, MD) using photomicrographed images. In this study, the following evaluation parameters were created: 1) bone area fraction around implants: the ratio of the bone area of interest (AOI) occupied in the tissue AOI (BAF [%]), 2) osteocyte density: the number of osteocytes in each AOI (osteocyte density [$\#/\text{mm}^2$]), 3) osteoclast number: the number of osteoclasts per bone perimeter in each AOI (N.Oc/BS [$\#/\text{mm}$]), 4) collagen area fraction: the ratio of stained area occupied in the tissue AOI (CAF [%]), and 5) production of type I and III collagen fibers: yellowish and greenish areas occupied in the tissue AOI (type I collagen [%] and type III collagen [%], respectively) [10]. AOIs were defined as the total inside and outside areas of the first, second, third and fourth threads.

Osteoblasts on bone surrounding the implants were visualized using immunostaining. Sections were deparaffinized, reacted with blocking agents, and incubated with primary antibody specific for Runx-related transcription factor 2 (Runx2) at 4 °C overnight. Rabbit anti-rat Runx2 polyclonal antibody was used as the first antibody (1:800, ab23981; Abcam, Cambridge, MA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (FUJIFILM Wako Pure Chemical Co), and the sections were then incubated with secondary antibody for 60 min at room temperature, developed with 3,3-diaminobenzidine (Sigma–Aldrich), counterstained using hematoxylin, and finally mounted. Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was used as the secondary antibody (ab6721; Abcam). The stained sections were photomicrographed using a light microscopy (Axio Scope A1; Zeiss) to quantitatively assess osteoblasts within the AOIs. The number of osteoblasts per bone perimeter in each AOI was semi-automatically counted (N.Ob/BS [$\#/\text{mm}$]) (Fig. 1C).

2.4. Statistical analyses

Normality was confirmed using the Shapiro–Wilk test. Nonparametric data were not noted. Student's *t*-test was used to compare the parametric data for the VC and PTH groups. All data are expressed as means \pm SEM. A *P*-value <0.05 was considered statistically significant. Systat 13.2 (Systat Software, Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Effects of intermittent subcutaneous administration of PTH on long bones

Intermittent PTH administration significantly increased Tb.BV/TV ($P = 0.003$), Tb.Th ($P = 0.035$) and Tb.BMD ($P = 0.018$), and this treatment significantly decreased Tb.Sp ($P = 0.004$) compared to

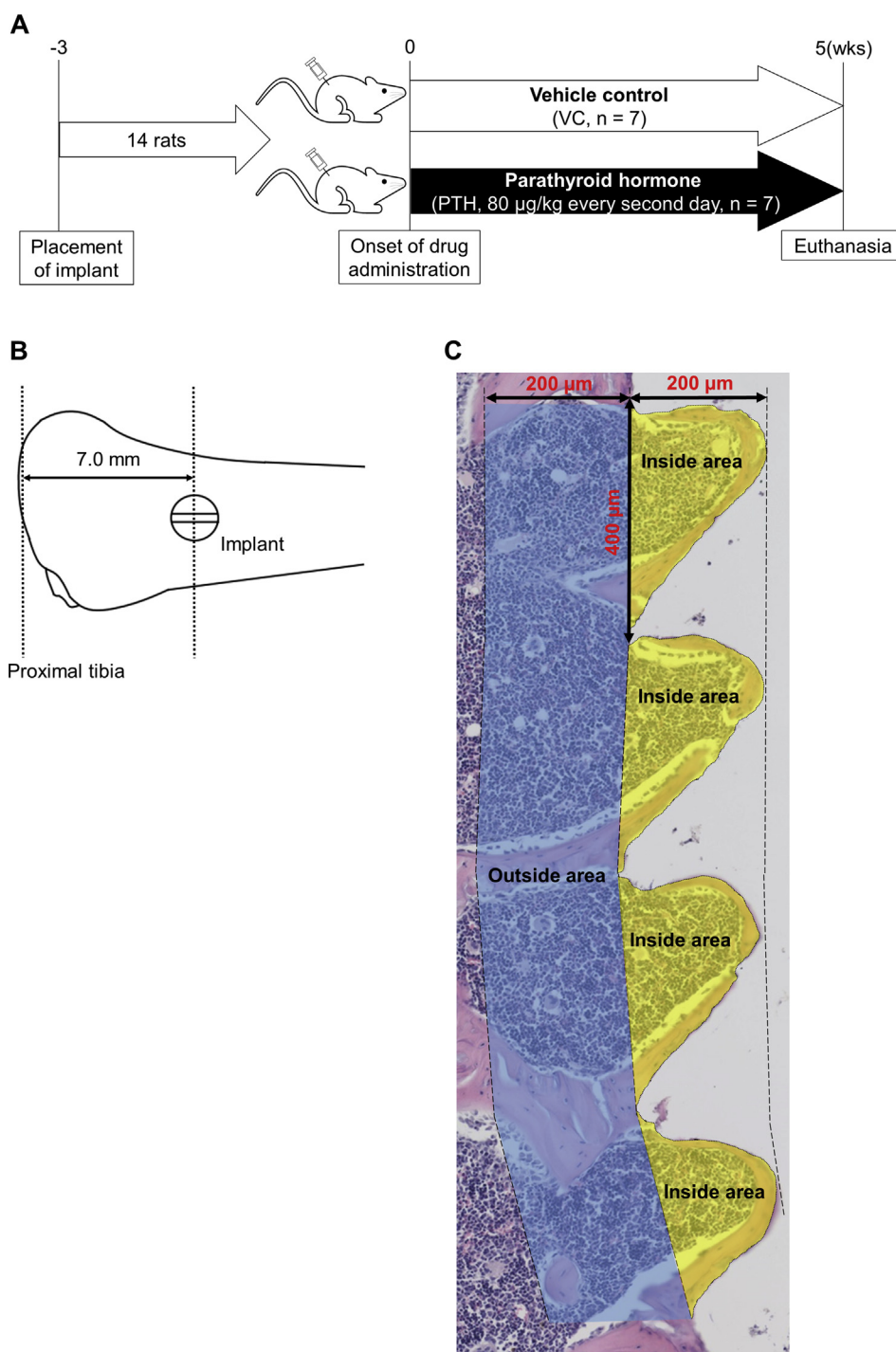


Fig. 1. Experimental schedules, surgery, and area of interests (AOIs). A) Time schedule for investigating the effects of intermittent PTH administration on bone quality and quantity surrounding implants. Euthanasia was performed at 5 weeks after drug administration. B) Placement position of implants. C) AOIs in the inside and outside areas of all implant threads.

those of the VC group (Fig. 2A–F). Intermittent PTH injection significantly increased Cv/Av ($P = 0.000$) and Ct.Th ($P = 0.009$) (Fig. 2G and H). Moreover, this treatment decreased Ct.In.length; however, the difference was not statistically significant ($P = 0.081$) (Fig. 2I). No change was observed in Ct.Ex.length between groups ($P = 0.554$) (Fig. 2J).

3.2. Effects of intermittent subcutaneous administration of PTH on bone quantity surrounding implants

Next, the effects of intermittent PTH injection on bone quantity based on bone area and the amount of collagen surrounding the implants were investigated. Intermittent PTH administration

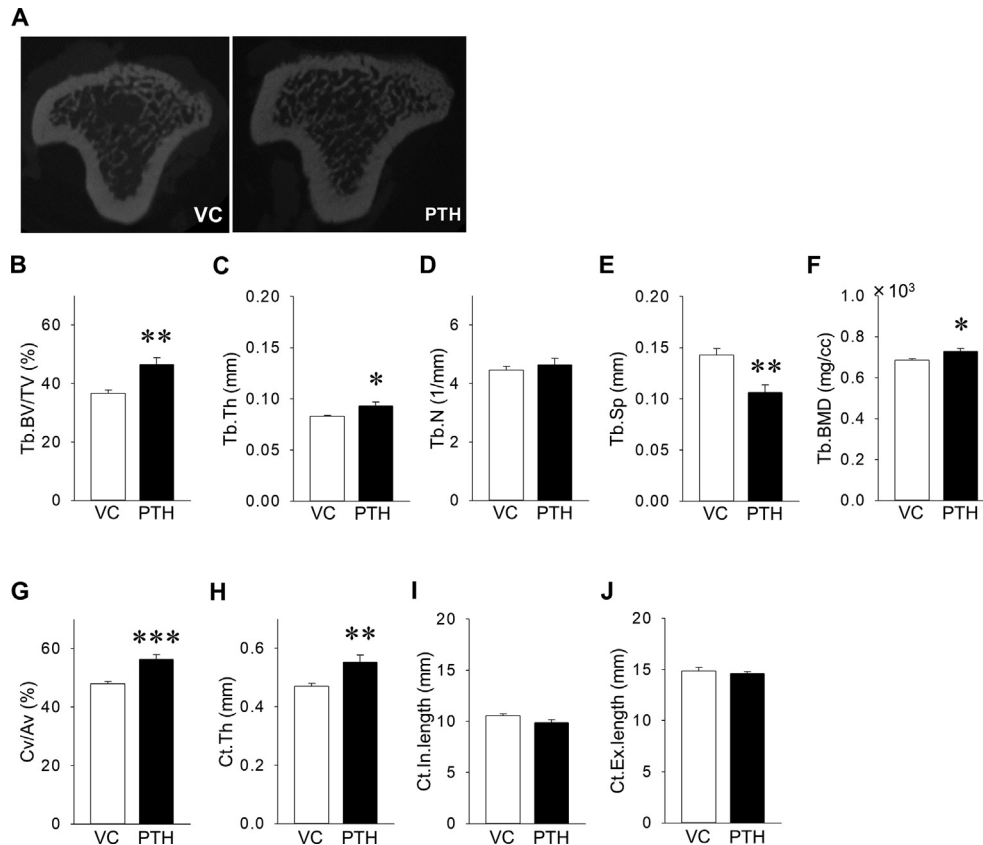


Fig. 2. Effects of intermittent PTH administration on long bones. A) Representative images of microCT-constructed cross-sectional images. B) Trabecular bone volume per tissue volume (Tb.BV/TV) was significantly increased in PTH vs. VC. C) Trabecular thickness (Tb.Th) was significantly increased in PTH vs. VC. D) Trabecular number (Tb.N) was the same between groups. E) Trabecular separation was significantly decreased in PTH vs. VC. F) Trabecular bone mineral density (Tb.BMD) was significantly increased in PTH vs. VC. G) Cortical volume ratio (Cv/Av) was significantly increased in PTH vs. VC. H) Cortical thickness (Ct.Th) was significantly increased in PTH vs. VC. I) Inner length of cortical bone (Ct.In.length) was decreased in PTH vs. VC, but the difference was not statistically significant. J) Outer length of cortical bone (Ct.Ex.length) was almost the same between groups. Graphs show means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test); $n = 7$ rats/group.

significantly increased both BAF and CAF in the total inside areas of all implant threads compared to levels in the VC group ($P = 0.043$ and $P = 0.023$, respectively) (Fig. 3A–C); however, this treatment resulted in no significant effect on BAF and CAF in the total outside areas of all threads compared to that in the VC group ($P = 0.995$ and $P = 0.260$, respectively) (Fig. 3A, D and E).

3.3. Effects of intermittent subcutaneous administration of PTH on bone quality based on the types of collagen surrounding the implants

Next, the effects of intermittent administration of PTH on bone quality based on the types of collagen surrounding the implants were investigated. Intermittent injection of PTH exerted no effect on the amount of type I collagen in the total inside area of all implant threads ($P = 0.433$); however, it did increase the amount of type III collagen compared to that in the VC group, although the difference was not statistically significant ($P = 0.094$) (Fig. 4A–C). Moreover, intermittent administration of PTH significantly increased type I collagen in the total outside area of all implant threads compared to that observed after VC administration ($P = 0.002$); however, this treatment did not affect type III collagen ($P = 0.379$) (Fig. 4A, D and E).

3.4. Effects of intermittent subcutaneous administration of PTH on bone quality based on bone cells surrounding the implants

Bone cells such as osteocytes, osteoclasts and osteoblasts surrounding the implants were examined. PTH administration

significantly increased the number of osteocytes in both the total inside and outside areas of all implant threads compared to that in the VC group (both $P = 0.000$) (Fig. 5A–C). Counting the visualized osteoclasts on the bone surfaces revealed that PTH administration significantly increased N.Oc/BS in the total inside areas of all implant threads compared to that observed after treatment with the VC ($P = 0.004$); however, this treatment did not affect the number of osteoclasts in the total outside areas of all implant threads ($P = 0.844$) (Fig. 5D–F). Osteoblasts on the bone surface were immunostained and then counted. Intermittent administration of PTH significantly increased N.Ob/BS in both the total inside and outside areas of all implant threads compared to the values observed after VC administration ($P = 0.039$ and $P = 0.025$, respectively) (Fig. 5G–I).

4. Discussion

We demonstrated that intermittent administration of PTH for 5 weeks improved not only bone quantity, but also the updated concept of “bone quality” around implants by affecting bone cells such as osteocytes, osteoclasts, and osteoblasts and also the types of collagen fibers in rat tibiae.

In this study, implants were placed into rat tibiae rather than the jawbone. The tibial bones of large and small animals have been extensively used to evaluate bone quantity based on bone volume surrounding implants [21]. However, the use of bigger animals such as beagle dogs and rabbits is challenging for molecular-level studies such as protein and gene analyses due to the limited number of

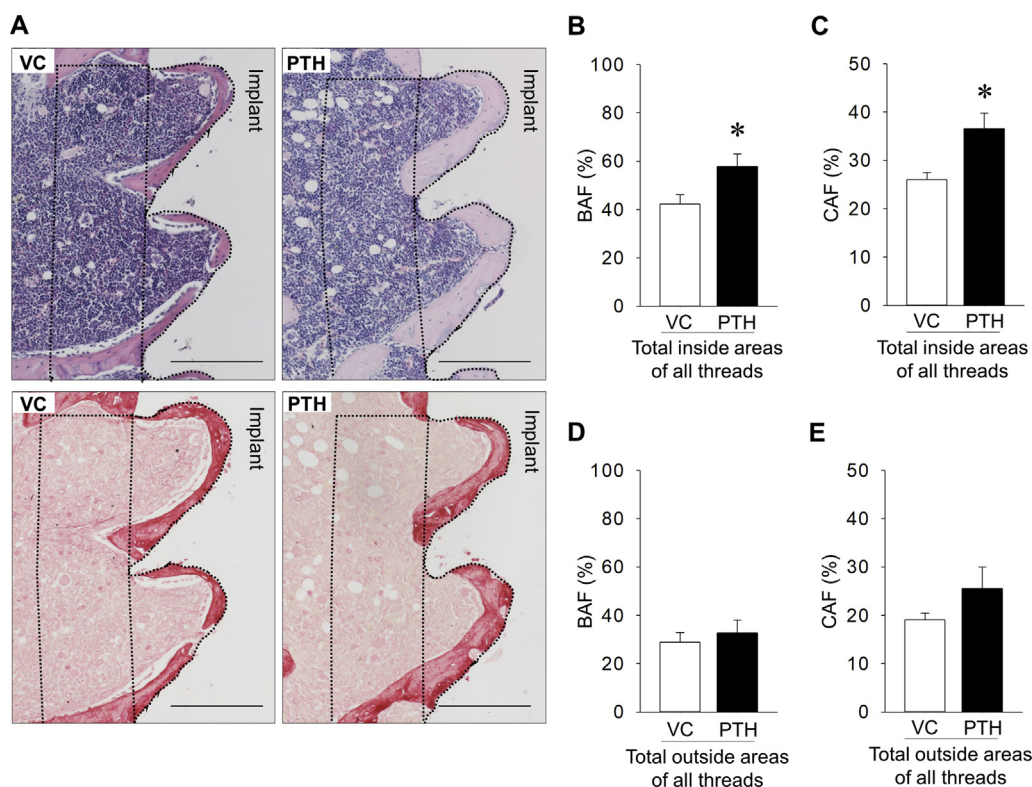


Fig. 3. Effects of intermittent PTH administration on bone quantity around the implants. A) Representative images of the inside and outside areas of first and second implant threads from H&E and picosirius red-stained sections (bar: 200 μ m; dotted line: AOIs in the inside and outside areas). B) Bone area fraction (BAF) in the total inside areas of all implant threads was significantly increased in PTH vs. VC. C) Collagen area fraction (CAF) in the total inside areas of all implant threads was significantly larger in PTH vs. VC. D) BAF in the outside areas of all implant threads was the same between PTH and VC. E) CAF in the total outside areas of all implant threads was similar in PTH vs. VC. Graphs show means \pm SEM. * $P < 0.05$ (Student's *t*-test); $n = 7$ rats/group.

specific antibodies and primers for these animals, although normal diameter and length of implants can be used in studies involving larger animals. Thus, in the present study, rats were used to investigate bone quality based on bone cells and the types of collagen fibers.

Clinically, PTH is subcutaneously administered daily to treat osteoporosis. In this study, 80 μ g/kg of human PTH(1–34) was subcutaneously administered to rats every second day for 5 weeks. In a previous study, subcutaneous injection of 80 μ g/kg of human PTH(1–34) every other day significantly increased bone volume and BMD, increased Tb.Th and Tb.N, and decreased Tb.Sp in C57BL/6J mice, although the anabolic effect was smaller than that of daily injection of the same dose [22]. Indeed, our findings in regard to structural alteration of the long bones by intermittent administration of PTH were partially supported by those of previous studies [23,24], as administration duration and frequency were different compared to those in the present study (4 weeks and 5 times per week in mice [23], and 6 weeks and daily injection in rats [24], respectively). However, as mentioned above, the administration frequency, route, and dosage of human PTH(1–34) in the present study were all appropriate for evaluating the effects of PTH on bone quantity and quality surrounding implants in rat tibiae.

Intermittent injection of 40 μ g/kg PTH for 8 weeks has been reported to enhance osseointegration and bone volume with increased Tb.Th around titanium implants placed in the tibiae of osteopenic rats that were fed a low protein diet [25]. In this previous study, interfaces between the implant and bone were excluded from the ROIs to avoid the presence of artifacts adjacent to implants caused by microCT [25]. It has been reported that detailed

validation studies are required when metal implants are used, since they may lead to metal artifacts by microCT scan and analyses [26]. It is possible that negative effects on bone quantity around implants using microCT could be observed as a result of artifacts in the present study. Thus, histomorphometric analyses rather than microCT analyses were used to accurately evaluate bone quantity surrounding the implants. Moreover, the AOIs were separated into the inside and outside areas of all implant threads to more thoroughly investigate bone quantity and quality. Our results demonstrated that intermittent administration of PTH significantly increases bone area and also increases collagen content in the total inside areas of all implant threads while not affecting the total outside areas. Therefore, previous data partially support our results regarding bone quantity around implants, as in the previous study, the ROI represented a combination of the total inside and outside areas of the implant threads [25]. Our new findings suggest that the anabolic effect on bone quantity surrounding implants strongly depends on bone in the inside area of the implant thread rather than that in the outside area. The ratio of collagen content in bone tissue may be associated with increased bone area induced by the intermittent administration of PTH.

The concept of “bone quality” was updated 20 years ago by the NIH [5]; however, recognition of its importance has been impeded as BMD is believed to be equivalent to bone quality in implant treatment. Consequently, there are limited data on “bone quality” surrounding implants in implant dentistry. The present study thus focused on the updated concept of “bone quality” around implants as assessed based on the types and abundance of bone cells and collagen fibers. First, differences in the types of collagen were

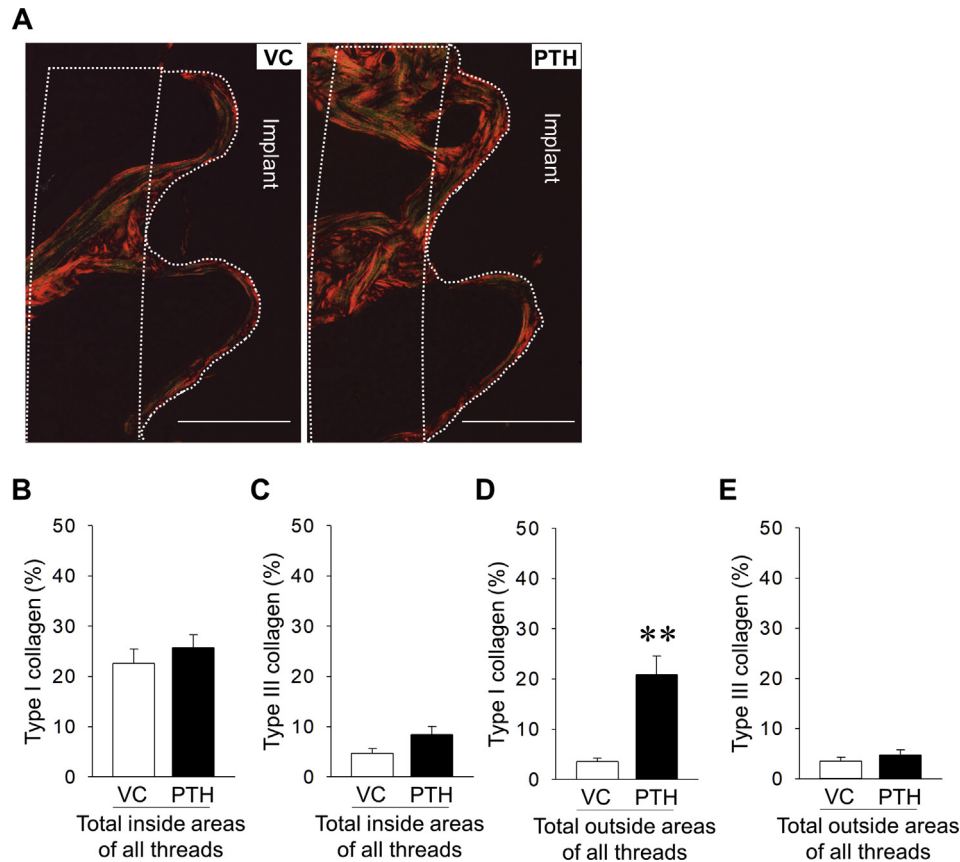


Fig. 4. Effects of intermittent PTH administration on bone quality based on collagen type surrounding the implants. A) Representative images of the inside and outside areas of implant threads from picrosirius red-stained sections (bar: 200 μ m). B) Production of type I collagen in the total inside areas of all implant threads was the same between groups. C) Production of type III collagen in the total inside areas of all implant threads was higher in PTH vs. VC, but the difference was not statistically significant. D) Production of type I collagen in the total outside areas of all implant threads was significantly larger in PTH vs. VC. E) Production of type III collagen in the total outside areas of all implant threads was the same between groups. Graphs show means \pm SEM. ** $P < 0.01$ (Student's *t*-test); $n = 7$ rats/group.

investigated using picrosirius red staining and polarized microscopy [27] due to the knowledge that collagen is a key factor controlling bone quality [28]. Interestingly, our findings show that the ratios of type I and III collagen to total collagen were different between the total inside and outside areas of all implant threads. Intermittent PTH administration has been demonstrated to activate osteoblasts, resulting in the upregulation of type I collagen production [29–31]. In contrast, it has been reported that intermittent injection of human PTH(1–34) increased the ratio of type I collagen without altering the amount of type III collagen in bone matrix using picrosirius red staining and polarized light microscopy [32]. Therefore, our data on the induction of type I collagen production induced by intermittent PTH administration is in accordance with the previous study, although our findings differ slightly for type III collagen [32]. Type III collagen has been reported to play an important role in trabecular bone formation and maintenance by regulating osteogenesis induced by osteoblasts [33], and this suggests that both type I and III collagen are important factors for bone quality. Slight increases in type III collagen in the total inside areas of all implant threads may contribute to enhanced bone quality in this study, although it remains unknown why the ratio of type I and III collagen differed between the inside and outside areas. We speculate that these differences could be influenced by factors such as the frequency of administration, the route and dosage of PTH, the condition of the rats, and the chosen AOIs and analyzed sites (inside

vs. outside areas of implant threads). Implant surface characteristics may also contribute to these differences. Further studies investigating the molecular mechanisms of collagen production induced by the intermittent administration of PTH surrounding implants are required.

Next, bone cells such as osteoblasts, osteoclasts, and osteocytes were histomorphometrically and immunohistochemically assessed to evaluate bone quality around the implants. The intermittent administration of PTH has been demonstrated to increase bone formation [34] by enhancing the differentiation of osteoblast precursors and bone lining cells into osteoblasts [35,36] and mesenchymal stem cells into osteoblasts via protein kinase C δ [37]. Intermittent injection of PTH inhibits apoptosis of both osteoblasts and osteocytes [38], possibly increasing RANKL production by osteoblasts and osteocytes and leading to increases in osteoclast numbers [39,40]. Moreover, intermittent PTH administration has been shown to negatively regulate sclerostin, a protein that inhibits bone formation [41]. Based on these modes of action of intermittent PTH administration, these previous data clearly support our present findings that intermittent administration of PTH significantly increases the number of osteoblasts and osteocytes in bone surrounding implants. An increase in the number of osteoblasts may contribute to increased bone area induced by PTH in the total inside areas of all implant threads, and an increase in the number of osteocytes may contribute to the development of an osteocyte

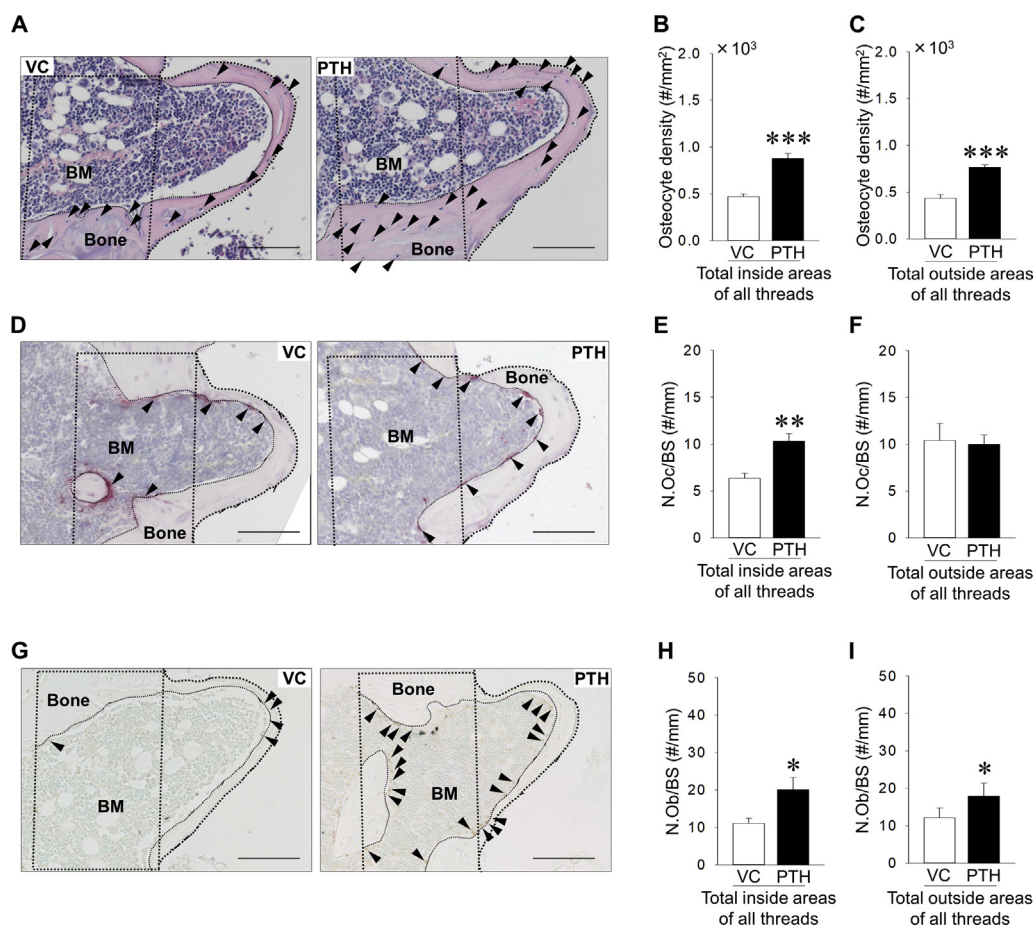


Fig. 5. Effect of intermittent PTH administration on bone quality based on bone cells around the implants. A) Representative images of the inside and outside areas of implant threads from H&E-stained sections (bar: 100 μ m; black arrow heads: osteocytes; BM: bone marrow). B) Osteocyte density in the total inside areas of all implant threads was significantly increased in PTH vs. VC. C) Osteocyte density in the outside areas of all implant threads was significantly increased in PTH vs. VC. D) Representative images of the inside and outside areas of implant threads from TRAP-stained sections (bar: 100 μ m; black arrow heads: osteoclasts on bone surface; BM: bone marrow). E) Osteoclast number (N.Oc/BS) in the total inside areas of all implant threads was significantly increased in PTH vs. VC. F) N.Oc/BS in the outside areas of all implant threads was similar between groups. G) Representative images of the inside and outside areas of implant threads from Runx2-immunostained-sections (bar: 100 μ m; black arrow heads: osteoblasts; BM: bone marrow). H) Osteoblast number (N.Ob/BS) in the total inside areas of all implant threads was significantly increased in PTH vs. VC. I) N.Ob/BS in the outside areas of all implant threads was significantly increased in PTH vs. VC. Graphs show means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test); $n = 7$ rats/group.

network that is important for handling mechanical loads such as masticatory and parafunctional loads around implants. Conversely, it was recently reported that intermittent human PTH(1–34) administration worsened bone quality as assessed by collagen integrity in mandibular regenerative bone but not in long bones [42]. Therefore, caution is required in interpreting our results that intermittent injection of PTH improves bone quality based on collagen and bone cells. It is unclear why osteoclast number increased in the total inside but not in the outside areas of all implant threads. The production of different collagen types in the total inside and outside areas of all implant threads may be linked to the changes in osteoclast number induced by intermittent administration of PTH.

5. Conclusions

Within the limitations of this study (e.g., PTH administration protocol, anatomical factors, analysis methods, and methodology to

evaluate bone quality), we demonstrated that subcutaneous intermittent administration of PTH significantly improved both bone quantity and bone quality based on the types and populations of bone cells and the types of collagen fibers surrounding the implants. We also demonstrated that the effects of PTH on bone quality in the total inside areas of all implant threads are larger than those in the total outside areas of all implant threads. The administration of PTH as an implant treatment may contribute to successful clinical outcomes due to improved bone quantity and quality surrounding dental implants.

Ethical statement

The animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Ethics Committee for Animal Research (Approval numbers 1608091331–2 and 1804181448–5).

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

Yusuke Uchida: Data curation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Shinichiro Kuroshima:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing - original draft, Writing - review & editing. **Yusuke Uto:** Software, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Riho Kanai:** Investigation, Visualization, Writing - original draft, Writing - review & editing. **Maaya Inoue:** Investigation, Visualization, Writing - original draft, Writing - review & editing. **Masayoshi Suzue:** Software, Investigation, Writing - original draft, Writing - review & editing. **Takashi Sawase:** Funding acquisition, Resources, Supervision, Writing - original draft, Writing - review & editing.

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