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Original Research

Evaluation of therapeutic effects of teriparatide in a rat model of zoledronic acid-induced bisphosphonate-related osteonecrosis

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ABSTRACT

Osteonecrosis of the jaw is a complication in patients using bisphosphonate agents. In this study, we aimed to investigate the effects and mechanism of teriparatide (TPTD) in a rat model of bisphosphonate-related osteonecrosis (BRON). Osteonecrosis was induced by administration of zoledronic acid (ZOL). Four weeks after ZOL injection, the rats underwent a surgery in which drilling holes were made. These holes were filled with freeze-dried *Aggregatibacter actinomycetemcomitans*. After the four-weeks period, TPTD or saline (n = 9) was intermittently administered for four weeks. Later, rats were euthanized, and the mandible and femur bones were examined the amount of necrosis and bone regeneration. Serum receptor activator of NF-κB ligand (RANKL), osteocalcin (OC), and C-terminal crosslinking telopeptide of type I collagen (CTX) were also examined. TPTD administration reduced necrotic bone area of the mandibles and femurs in the BRON rat model and induced new bone formation. In addition, TPTD injection increased the number of osteoclasts. The suggested underlying mechanism is the induction of protein levels of RANKL by TPTD. Furthermore, the serum levels of bone metabolism biomarkers (OC and CTX) were upregulated in the TPTD injection group. In conclusion, ZOL has negative effects on osteoclasts. TPTD was found to be effective in eliminating the negative effects of ZOL. TPTD had positive effects in preventing bone resorption and promoting osteogenesis. In addition, TPTD improved osteoclastogenesis, which in turn led to the improvement of BRON.

1. Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a rare but serious complication in patients using nitrogen-containing bisphosphonates (NBPs) after tooth extraction to prevent or treat metastatic cancer, Paget's disease, multiple myeloma, hypercalcemia, and osteoporosis [1]. Initially, osteonecrosis was reported only after treatment with bisphosphonates and referred to as BRONJ. However, as other antiresorptive agents (for example, monoclonal antibodies such as denosumab) seemed to lead to the same phenomenon as NBPs, the terminology was soon changed to antiresorptive-related osteonecrosis of the jaw (ARONJ) [2]. Since 2014, the American Association of Oral and Maxillofacial Surgeons (AAOMS) has recommended the use of the term "medication-related osteonecrosis of the jaw" (MRONJ) [3]. Because the cases of osteonecrosis of the jaw have increased in patients undergoing other antiresorptive and anti-angiogenic treatments, the name was changed from BRONJ to ARONJ and MRONJ. Regarding the treatment of ARONJ, MRONJ, and BRONJ, in accordance with the AAOMS position paper, conservative treatment is recommended for BRONJ [3]. Nevertheless, some cases showed resistance to these conservative treatments. Even after almost 10 years, this topic is still not well understood, leading to significant impairment of quality of life in these patients.

In contrast, teriparatide (TPTD) (Forteo®; Eli Lilly, IN, USA and Teribone®; Asahikasei, Tokyo, Japan), a synthetic polypeptide hormone that contains the 1–34 amino acid fragment of recombinant human parathyroid hormone (PTH), is a new synthetic PTH that has been recently used to improve the thickness and quality of the bone. Daily and

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weekly administration of TPTD reduces the risk of osteoporotic bone fractures. It is an alternative to anti-osteoporosis drugs and anti-resorptive agents. TPTD administration stimulates new bone formation at the initial phase, followed by bone remodeling and increase in bone mineral density. Daily subcutaneous injection (Forteo®) and weekly subcutaneous injection (Teribone®) formulas of TPTD are available as anabolic agents. The development of these injection formulas is anticipated to lead to new therapeutic methods for patients with osteoporosis at a high risk of fragile fracture [4]. In 2007, Harper and Fung reported cases where TPTD therapy was successfully used for the treatment of BRONJ [5]. Additionally, some hospital facilities reported that bisphosphonate-induced pathological fracture of the mandible was cured by TPTD administration [6,7]. Several subsequent studies also demonstrated that daily and weekly TPTD supportive conservative treatment was effective for the short-term treatment of BRONJ [8–10]. However, there is not enough evidence on the effects of the intermittent administration of TPTD after the occurrence of BRONJ. To address this problem, in this study, we hypothesized that TPTD usage would be beneficial in cases of BRONJ, and we investigated whether TPTD administration would have a curative effect on BRON model rats. TPTD was administered using a protocol designed to mimic as closely as possible the clinical use of this drug in humans. In addition, we investigated the phenotypes of osteoblasts and osteoclasts to elucidate the mechanism of bone repair following osteonecrosis induced by NBPs.

2. Material and methods

2.1. Animals and experimental study design

The experimental protocol was approved by the Kyushu Dental University Committee on the Use and Care of Animals (May 18, 2016 No.16-009). Eighteen male Wistar rats (8-week-old; body weight 288–316 g) were used in the experiments. All animals had free access to laboratory food and water. They were individually housed in plastic cages in a temperature-controlled room (23 °C) with a 12/12-hr light/dark cycle. In accordance with our previous animal studies [11], the rats were injected subcutaneously with zoledronic acid (ZOL) (Novartis Pharma, Basel, Switzerland) at a dose of 0.1 mg/kg of body weight weekly for 4 weeks. The dose of ZOL given was determined based on the dose used for the clinical treatment of cancer patients. One week after the final injection (13-week-old), the rats underwent a surgical procedure in which a drill hole was made in the bilateral mandibular borders and in the diaphysis of bilateral femurs. In these experimental procedures, the animals were placed under general anesthesia in a supine position. After shaving, the skin over the mandibles and femurs was cut bilaterally, and a blunt dissection over the bone was performed. The periosteum of the mandibular border and femur was cut, and then reflected in a longitudinal direction along the bone. Next, the length and width were measured, and a small bone socket 1.8 mm deep was drilled through the cortical bone in the posterior portion of the mandibular border and in the center of the ventral surface of the femur using a 1.8-mm diameter round bur with sufficient irrigation. After making bilateral drilling holes, a microfibrillar collagen hemostat (ZERIA Pharmaceutical, Tokyo, Japan) soaked with freeze-dried *Aggregatibacter actinomycetemcomitans* (40 μg/site, dissolved in 4 μL saline), which produces bacterial toxins, was placed into each bone hole according to the method of Tsurushima et al. (2013). Finally, the drilling holes were covered with yellow bone wax (Alfresa Pharma Corporation, Osaka, Japan). After irrigation of the wounds, complete wound closure was performed. 0.5% meloxicam was injected (subcutaneous injection, 0.2 mg / kg) immediately after surgery and one day after surgery for postoperative pain. And 10% isosin is used to perform surgical disinfection of surgical field and thorough sterilization of wound site after surgery for the purpose of preventing postoperative infection. Four weeks after surgery, the rats were utilized as experimental animals and divided into two groups: TPTD group (n = 9), in which the rats were injected subcutaneously with TPTD (Teribone®; Asahikasei, Tokyo, Japan) at 30 μg/kg of body weight three times a week for 4 weeks [12] and saline group (n = 9), in which the rats were injected with an equal volume of saline using the same protocol. One week after these treatments, the rats were euthanized. To evaluate the dynamic parameters of new bone formation, the rats also received an intraperitoneal injection of 10 mL of calcein (SIGMA C-0875 15 mg/kg) dissolved in phosphate-buffered saline (PBS) containing 2% sodium bicarbonate 10 days and 3 days prior to sacrifice. The mandibles and femurs were removed, and decalcified specimens were prepared with the right mandible and femur, and non-decalcified specimens were prepared with the left mandible and femur. In the decalcified specimens, the retrieved bones were fixed in 10% buffered formalin solution for 24 h and then decalcified with 10% buffered formic acid solution for 48 h. After embedding with paraffin, thin sectioning was performed at a thickness of 5 μm, and hematoxylin-eosin (H-E) staining was performed. After fixation with 70% buffered ethanol solution, non-decalcified specimens were subjected to Villanueva bone staining for 2 weeks and then embedded in methyl methacrylate (Wako, 297-56001, Osaka, Japan) to prepare sections of 5-μm thickness. These stained sections were examined under a light microscope.

In the decalcified specimen, the necrotic bone area and new bone formation area of both groups were measured. We defined osteonecrosis as a region of empty lacuna > 500 mm^2 and new bone formation as a region of bone in the drilling socket in accordance with the definition of Allen and Burr (2008). The area of necrotic bone contiguous with the drilled site and the number of osteoclasts were measured using a DP2-BSW (Olympus, Tokyo, Japan). In the non-decalcified specimen, the width of double calcein labeling in the bone sections was measured at five arbitrary points, and the average value was calculated using cellSens (Olympus).

2.2. ELISA of serum bone biochemical markers

At the end of each experimental period, the animals scheduled for euthanasia at the specific time underwent terminal blood sampling by cardiac puncture under general anesthesia. Blood samples were allowed to clot for 2 h and centrifuged at 1000 ×*g* for 20 min at 4 °C. The prepared sera were assayed and the protein levels of receptor activator of NF-κB ligand (RANKL) were investigated by enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp., Houston USA). Serum osteocalcin (OC) and C-terminal crosslinking telopeptide of type I collagen (CTX) levels were measured by ELISA kit (Rat's-OC kit: GE Healthcare Japan Corp., Tokyo, Japan; Rat's-CTX kit: Immunodiagnostic systems Ltd., Boldon, UK). All samples were measured blinded and in triplicate according to the manufacturer's instructions and then averaged.

2.3. Statistical analysis

All data are presented as mean ± standard deviation (SD). Differences between the two groups were evaluated using the Welch's *t*-test (intergroup analysis). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Clinical findings

All the rats tolerated the experiment well. None of the rats exhibited mucosal ulceration, abscesses, fistula formation, or necrotic bone exposure in clinical examination.

3.2. Histopathological findings

3.2.1. New bone formation and necrotic bone area

In both mandibular and femoral sections in the saline group, a large

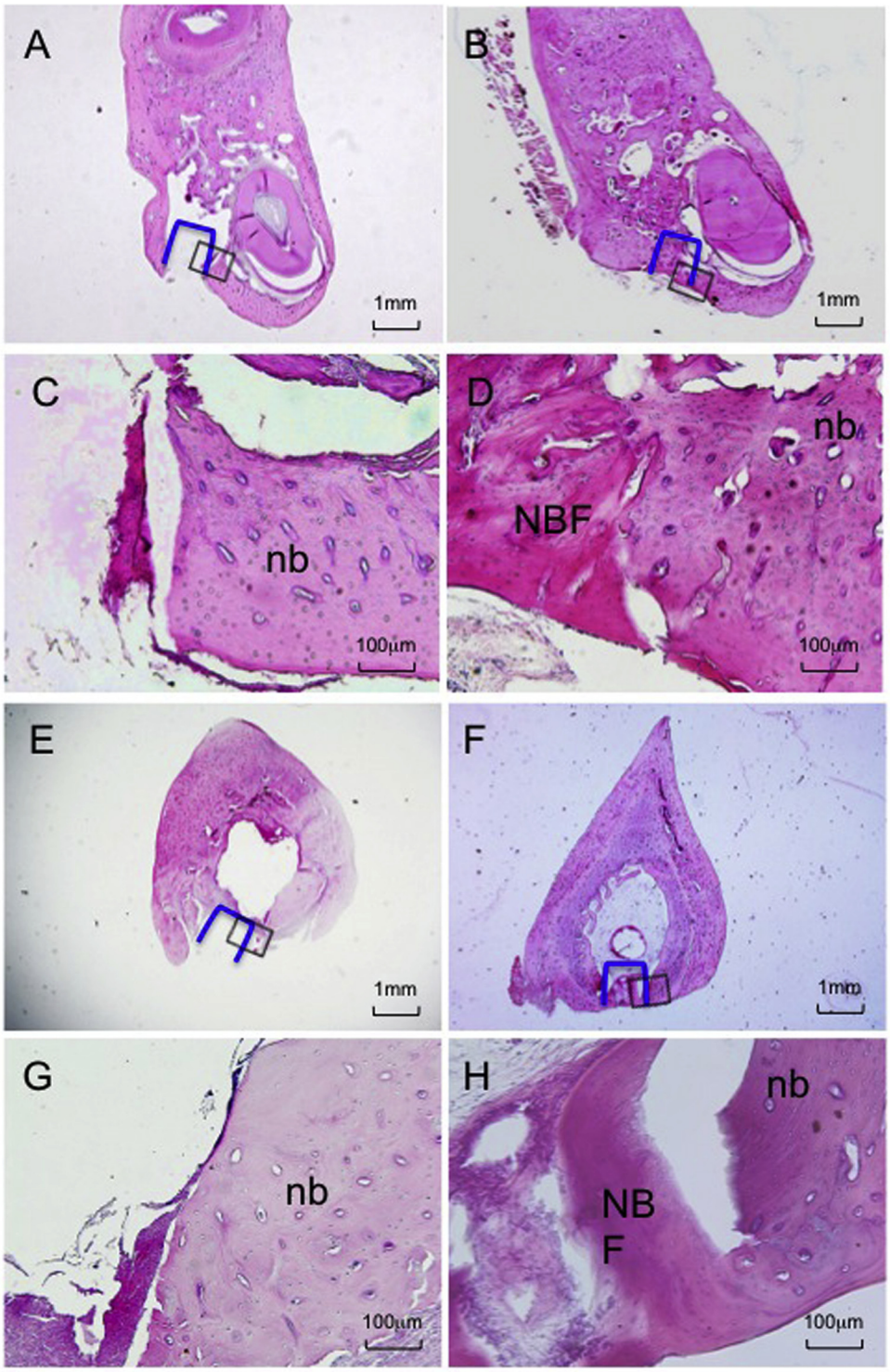

Fig. 1. Microscopic examination of hematoxylin-eosin staining of the mandible and femur. (A) In the saline group, bone cells disappeared in the bone lacuna around the drill hole, and osteonecrosis was confirmed. New bone formation was not observed in the drill holes of the cortical bone. (B) In the TPTD group, necrotic bone as well as new bone formation was observed in the drill holes. (C) Higher magnification of image in Fig. 1A (black frame). (D) Higher magnification of image in Fig. 1B (black frame). (E) In the saline group, as shown in the mandible section, new bone formation was not observed in the drill hole (blue line) and necrotic bone was observed in the surrounding of the drill hole. (F) In the TPTD group, necrotic bone area was small. New bone formation was observed as shown in the mandible section. (G) Higher magnification of image in Fig. 1E (black frame). (H) Higher magnification of image in Fig. 1F (black frame). (nb: necrotic bone, NBF: new bone formation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

area of osteonecrosis was formed around the drill holes compared with that in the control group (non-zoledronic-acid administration group), consistent with our previous study [11] and almost no new bone formation was observed in the perforated area (Fig. 1). In contrast, in the TPTD group, histological sections showed a healing phase in both mandible and femur. In both mandibular and femoral sections in the TPTD group, adequate new bone formation was observed in the drill holes and necrotic bone area was smaller than that in the saline group. Statistical comparison showed a significantly large area of newly formed bone and small area of necrotic bone in both the mandible and femur in the TPTD group ($P < 0.01$; Fig. 2).

3.2.1.1. Mandible. Microscopic examination of the drill holes showed that the area of newly formed bone was 0.14 ± 0.06 mm^2 in the saline group and 2.27 ± 0.46 mm^2 in the TPTD group. The area of necrotic bone was 1.63 ± 0.38 mm^2 in the saline group and 0.41 ± 0.12 mm^2 in the TPTD group (Fig. 2).

3.2.1.2. Femur. Microscopic examination of the drill holes showed that the area of newly formed bone was 0.14 ± 0.07 mm^2 in the saline group and 1.68 ± 0.37 mm^2 in the TPTD group. The area of necrotic bone was 2.52 ± 0.52 mm^2 in the saline group and 1.01 ± 0.33 mm^2 in the TPTD group (Fig. 2).

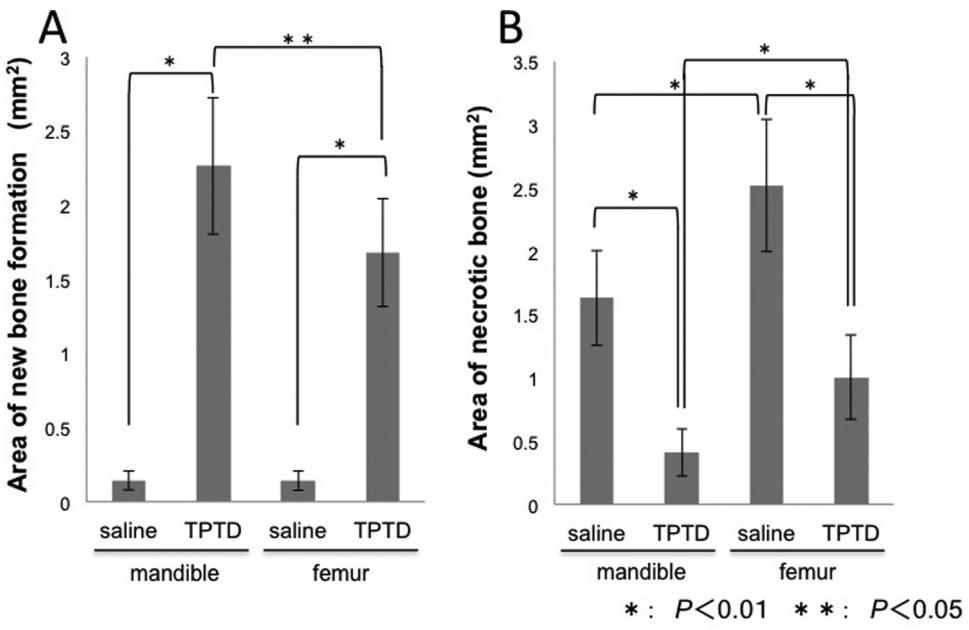

Fig. 2. Statistical analysis of hematoxylin-eosin staining of the mandible and femur, and measurement of new bone formation and necrotic bone area.
Increased new bone formation (A) and decreased necrotic bone (B) were observed in the TPTD administration group, with significant differences between both mandible and femur ($P < 0.01$).

3.3. Width of double calcein labeling

We measured the dynamic parameters of bone formation by double calcein labeling. A clear double line was confirmed in the TPTD group (Fig. 3). The double label line in both mandibular and femoral sections was significantly wider in the TPTD group than in the saline group ($P < 0.01$; Fig. 4).

3.3.0.1. Mandible

The width of double label of bone perforation was 5.31 ± 1.07 μm in the saline group and 32.90 ± 6.02 μm in the TPTD group (Fig. 4).

3.3.0.2. Femur

The width of double label of bone perforation was 5.14 ± 2.12 μm and 29.25 ± 6.31 μm in the TPTD group (Fig. 4).

3.4. Number of osteoclasts

The two sections were investigated, and the numbers of osteoclasts were compared using Villanueva bone staining (Fig. 5). A statistically significant difference in osteoclast numbers attaching to the necrotic bone was detected between the saline group and TPTD group. The numbers of osteoclasts in the TPTD group increased significantly compared with that in the saline group both in the mandibular and femoral sections ($P < 0.01$; Fig. 6).

3.4.1. Mandible

The numbers of osteoclasts were 2.67 ± 1.00 in the saline group and 8.11 ± 1.54 in the TPTD group (Fig. 6).

3.4.2. Femur

The numbers of osteoclasts were 4.22 ± 1.30 in the saline group and 12.11 ± 1.22 in the TPTD group (Fig. 6).

3.5. ELISA of bone biochemical markers

A statistically significant difference was observed in the level of RANKL protein between the saline group and TPTD group. We observed that RANKL level increased significantly in the TPTD group compared with that in the saline group ($P < 0.01$) (Fig. 7A). In addition, the measurements of serum bone metabolism markers (OC and CTX) demonstrated that both OC and CTX were upregulated after TPTD injection. There were significant differences between the saline and TPTD groups for OC and CTX, which are reliable indices for osteoblasts and osteoclasts, respectively ($P < 0.01$; Fig. 7B, C).

4. Discussion

In clinical practice, TPTD has been reported to be effective in treating BRONJ cases; however, there is insufficient evidence regarding clinical outcomes. In this study, we aimed to investigate the effects and mechanism of action of TPTD using a BRON rat model. We found that TPTD exhibited beneficial effects by reducing necrotic bone area and promoting osteogenesis. In addition, TPTD improved osteoclastogenesis, consequently leading to the improvement of BRON. The bone undergoes constant remodeling via new bone formation by osteoblasts and bone resorption by osteoclasts. The bone mass is maintained by a functional equilibrium state between these two cell types. Patients with osteoporosis are usually treated with bisphosphonates as a first-line therapy because of their medium to high effectiveness, long history of use, and cost effectiveness. Presently, ZOL is the most widely used agent for the management of osteoporosis and metastatic bone disease [13]. It is well-known that ZOL is an NBP, and NBPs promote osteoclast apoptosis and lead to decreased bone resorption and remodeling. Additionally, recent studies showed that NBPs have side effects such as BRONJ, atrial fibrillation, provocation of an acute-phase response, and renal failure [14,15]. These side effects may be caused by the inhibition of osteoclastogenesis and decreased activity and numbers of osteoclasts and other types of cells (for example, immune cells) [16]. Recent studies have reported that NBPs inhibit osteoblast activity and number [17]. Moreover, NBPs inhibit osteoclastogenesis and osteoblastogenesis. Taken together, these results suggest that NBPs decreased bone resorption and bone remodeling. The detailed mechanism of inhibition of bone resorption and bone remodeling by NBPs is not well understood. It is well-known that dentoalveolar procedures in patients exposed to NBPs greatly increase the risk of osteonecrosis of the jaws. No standard strategy for prevention of this disease has been established [3,9]. To date, it was believed that surgical procedures cause MRONJ, including BRONJ; however, recent research including our reports revealed that bacterial infection, in particular lipopolysaccharide (LPS), is the most important trigger of BRONJ onset [11,18]. Recently, there have been reports, including our report, on the beneficial effects of TPTD therapy in the prevention and treatment of BRONJ [6–9,19,20].

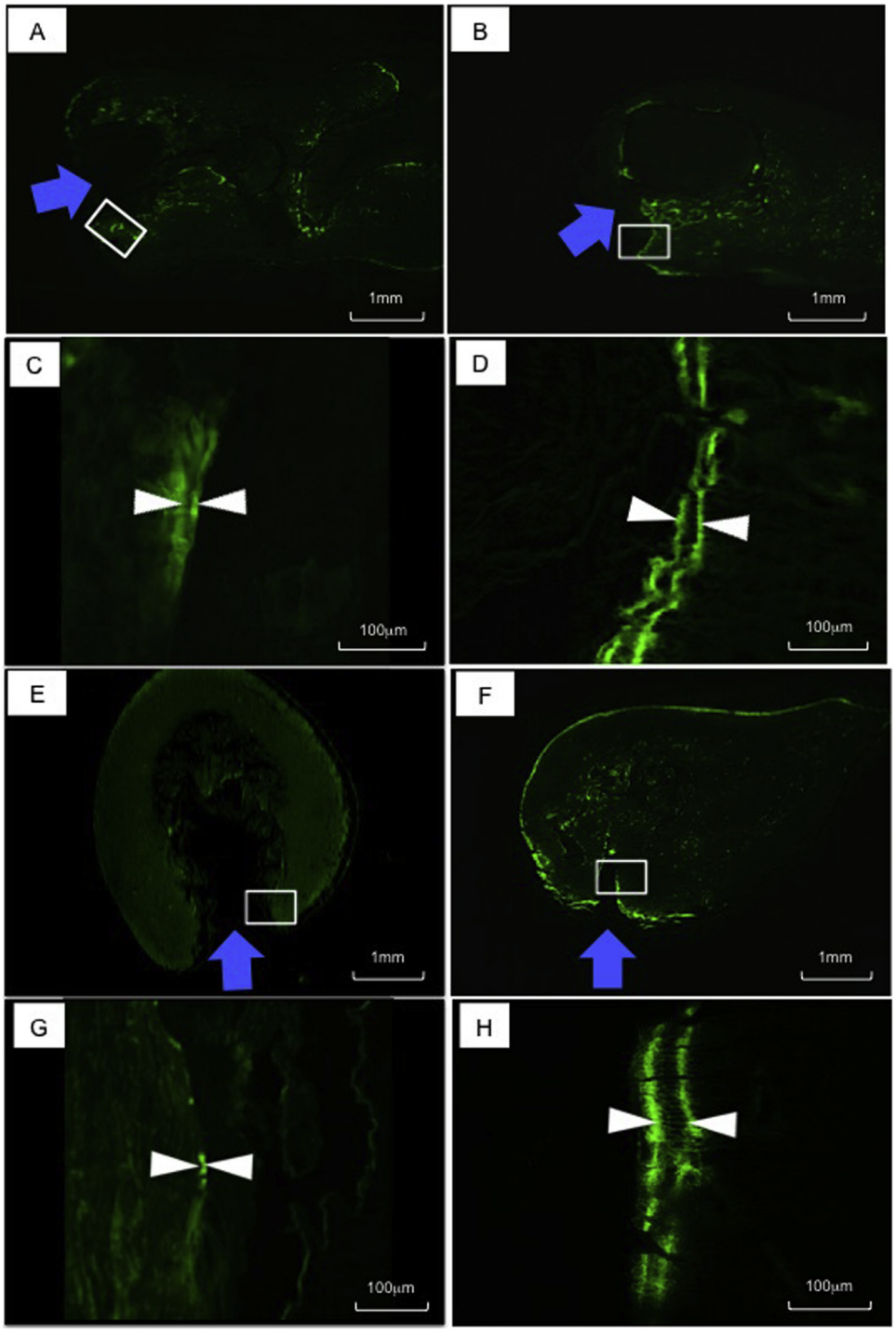

Fig. 3. Microscopic examination of double calcein staining of the mandible and femur. Representative confocal images showing calcein labeling in the callus of drill holes after 2 weeks of surgical treatment. (A) In the saline group, faint line (green) was observed (white arrow); however, this was not observed in the drill hole (blue arrow) at 2 weeks after operation. (B) In the TPTD group, the double labeling line (wide) was clearly observed in the drill hole, confirming that new bone formation increased in the drill hole at 2 weeks after operation. (C) Higher magnification of image in Fig. 3A (white frame). (D) Higher magnification of image in Fig. 3B (white frame). As shown in the mandible specimen, (E) calcein label (indicated by faint green line) was observed (white arrow) in the saline group; however, this was not observed in the drill hole (blue arrow). (F) In the TPTD group, the double labeling line (wide) was clearly observed with in the drilling hole. (G) Higher magnification of image in Fig. 3E (white frame). (H) Higher magnification of image in Fig. 3F (white frame). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Recombinant PTH [(rh PTH 1–34); Forteo® (teriparatide; Eli Lilly and Co, IN)] is approved by the United States Food and Drug Administration for the daily treatment of osteoporosis [21]. Additionally, Teribon® (teriparatide; Asahi Kasei Pharma Corp, Japan) is approved in Japan since November 2011 for the weekly treatment of osteoporosis. In agreement with the study by Tsurushima et al [11], our study also showed osteonecrosis in a BRON rat model in response to bacterial infection. This BRONJ model rat (Tsurushima et al., 2013) is the first capable of inducing osteonecrosis in the femur using bacterial infection and can induce osteonecrosis by 100%. Areas of osteonecrosis with empty osteocyte lacunae were observed surrounding the drill holes of the mandibles and femurs. This allows for quantitative evaluation, which is not possible in other model animals. In the TPTD-treated groups, new bone formation was observed in the drill holes of the mandibles and femurs with minimal necrotic bone (empty osteocyte lacunae) at 8 weeks after operation. An increase in osteoclast number, both in the mandibular and femoral sections, was observed in the TPTD groups but not in the control groups by Villanueva bone staining. The effect of PTH on osteoclastogenesis in rats has been reported previously [22]. Similarly, our study showed the effect of TPTD on osteoclastogenesis in a rat model.

PTH has been reported to stimulate RANKL gene expression [23]. RANKL, produced by osteoblasts, is a transmembrane ligand that can bind to RANK. RANK/RANKL interaction can initiate gene expression and signaling cascade that can lead to the differentiation and maturation of osteoclast precursor cells to mature osteoclasts [24–27].

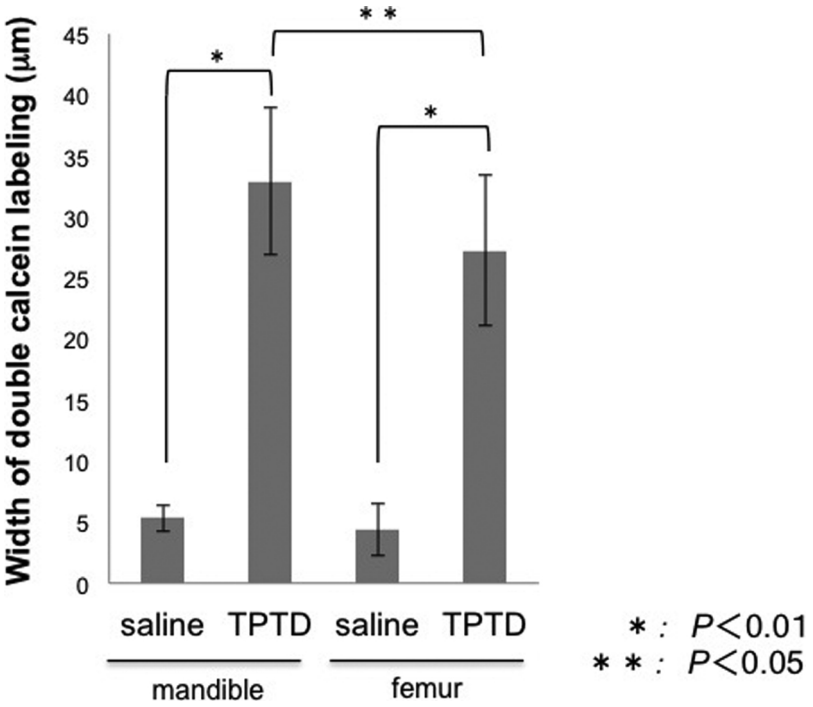

Fig. 4. Statistical analysis of double calcein staining of the mandible and femur, and measurement of new bone formation.
The double calcein labeling in both mandibular and femoral sections was significantly wider in the TPTD group than in the non-TPTD group ($P < 0.01$).

Furthermore, evidence suggests that the main mechanism by which PTH stimulates osteoclastogenesis is via increasing the expression of RANKL [28–31]. The serum levels of RANKL in postmenopausal patients remained unchanged even after the administration of TPTD [32]. Therefore, RANKL levels after TPTD treatment was controversial. In the present study, the serum level of RANKL was higher in the TPTD group than in the non-TPTD group. Moreover, our data showed that the serum osteoclast bone marker CTX and the osteoblast bone marker OC were upregulated in the TPTD group. From these results, we can suggest that osteoblasts were activated by TPTD administration and induction of RANKL expression resulted in the induction of osteoclasts in a BRON rat model. Marx et al evaluated the risk of BRONJ based on serum CTX [33]. This technique may help to detect and evaluate the initial stages of BRONJ. Previously, we also reported the relevance of NTX, an osteoclast marker similar to CTX, to the evaluation of refractory BRONJ [34]. In the present study, we evaluated the effect of TPTD treatment on BRONJ by measuring serum CTX, and our result confirmed a previous report [35]. A current concept in the mechanism of TPTD function is

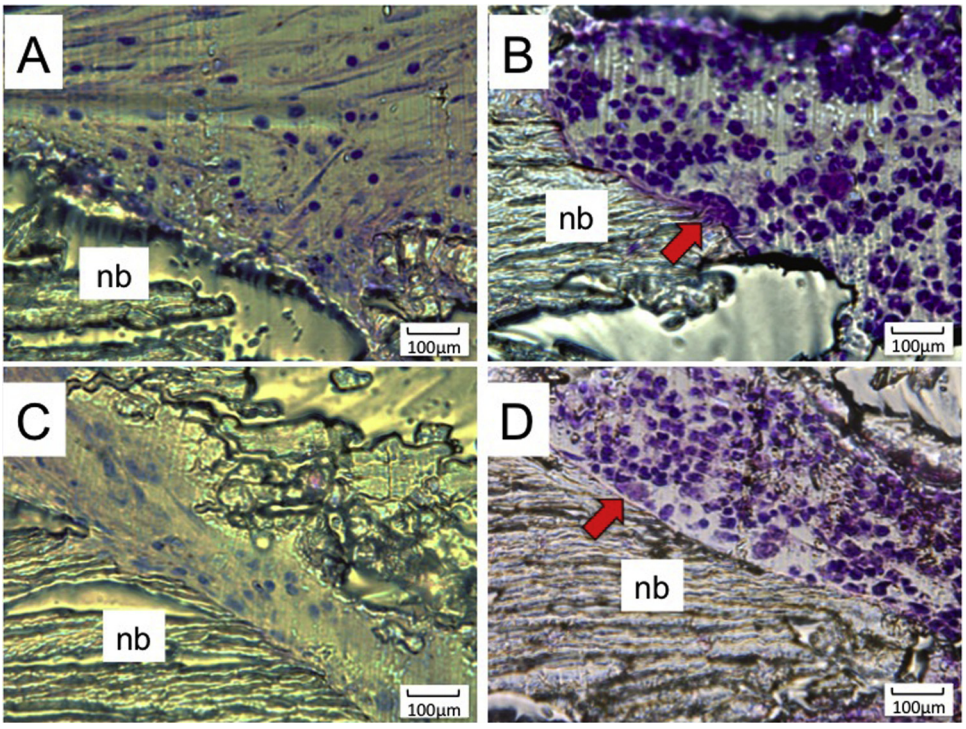

Fig. 5. Microscopic examination of Villanueva bone staining of non-decalcified specimens of the mandible and femur, and osteoclasts attached to the necrotic bone surface.
(A, C) In the saline group, almost no osteoclast-like cells were observed in both mandible and femur. (B, D) In the TPTD group, osteoclasts were observed on the surface of the necrotic bone in both mandible and femur (red arrows). (nb: necrotic bone). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

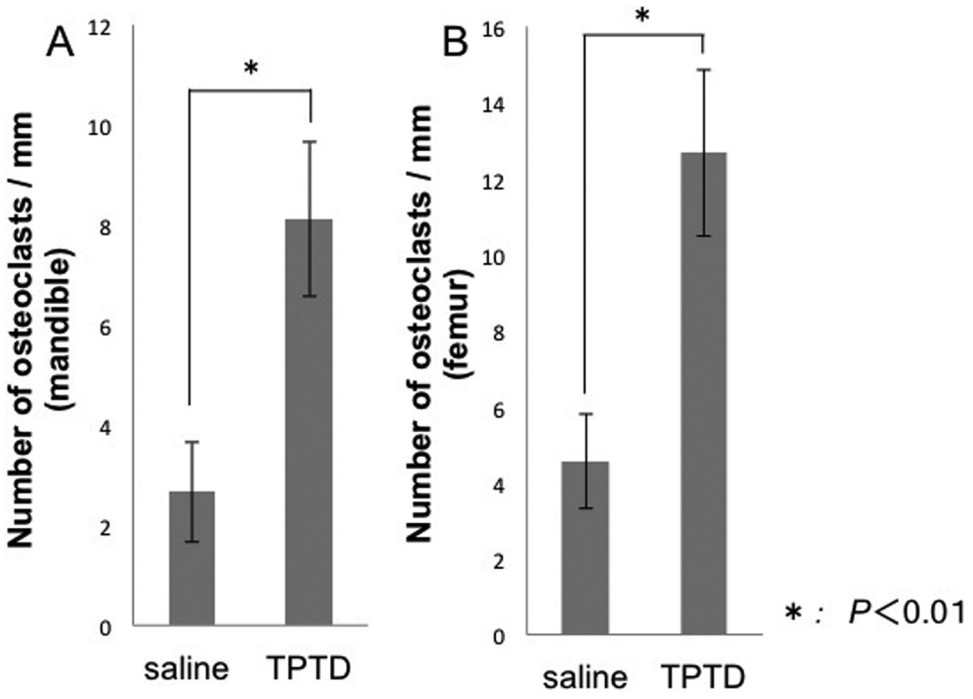

Fig. 6. Statistical analysis of Villanueva bone staining of the mandible and femur, and quantification of osteoclasts attached to the necrotic bone surface.
The number of osteoclasts attached to the necrotic bone surface was counted. The number of osteoclasts in both mandibular (A) and femoral (B) sections significantly increased in the TPTD group when compared with the saline group ($P < 0.01$).

related to its effect in stimulating processes associated with bone formation before the stimulation of processes related with bone resorption. This sequence of phenomenon has led to the concept of the "anabolic window," the period of time when TPTD is maximally anabolic. The improvement in BRONJ and induction of bone formation in our TPTD-treated rat model confirmed the theory of the "anabolic window" [36].

When the mandibular and femur sections were compared, it was observed that the necrotic bone area of the femur was significantly larger in the saline group ($P < 0.01$). This finding is in accordance with the result of Tsurushima et al. in BRON model [11]. It has been suggested that the femur bone has lower resistance to infection (inflammatory stimulus) than the mandible. Nevertheless, in clinical practice, most of the BRON is confined to the jaw. Tsurushima et al. concluded that the reason for the overwhelming majority of cases of osteonecrosis being limited to the jaws is because the thin oral mucosa covering the jaw bone can be broken easily. In addition, the existence of the teeth makes bacterial invasion of the jaw bone possible. It has been suggested that environmental factors typified by oral bacteria (especially LPS induced) are important factors for the onset of BRONJ [11,18,37]. In the TPTD group, the increase in newly formed bone area as well as the widening of double calcein labeling, and the decrease in necrotic bone area was significantly different in the mandible when compared with that in the femur bone ($P < 0.05$). Tim Van den Wyngaert et al. reported that BP exert a stronger effect on mandibular bone turnover relative to the femur, which strengthened the hypothesis that the inhibition of bone turnover may be important in the pathophysiology of ONJ. The results obtained in our experiment also show that the difference in the rate of turnover of the mandible influenced the results [38].

Among the mechanisms and treatment methods of BRONJ actively discussed, the concept of drug holiday attracts attention. This concept was reported in the position paper of AAOMS in 2014 [3]. However, these findings must be interpreted with caution because the paper reported the results of a retrospective research and the sample size was small. Patients using bone resorption suppressants for more than 4 years or osteoporotic patients having risk factors for osteonecrosis of the jaw who have been asked to undergo an invasive dental treatment should discuss with the attending physician whether a 2-month drug holiday would be adequate before an invasive dental procedure. In contrast, it has been reported that the risk of pathological fracture is increased by the 2-month withdrawal of BPs before a dental invasive treatment and the cure periods to epithelialization healing were occurred [39]. The frequency of BRONJ was not so high while taking bone resorption suppressants. Therefore, many studies have reported skeptical opinions about preventive drug holiday. Finkelstein et al. compared the effects of TPTD, alendronate, and their combination on osteoporosis and reported that TPTD showed the highest increase in bone mineral density [40]. Zandi et al. also reported that preoperative administration of TPTD has an effect on BRONJ prevention. They showed that 4 weeks of TPTD administration starting on the same day or 2 weeks before tooth extraction successfully reduced the incidence of BRONJ in rats treated with zoledronate [41]. Therefore, we propose the usefulness of substitution of TPTD during BP withdrawal period in preventing the occurrence of BRONJ. Further studies on the above findings are necessary for the clinical application of this strategy.

5. Conclusion

In our study, we showed that TPTD promotes osteoblast activity, thereby increasing the expression of RANKL in serum, which in turn promoted the activation of osteoclasts and consequently led to improvement of BRONJ. However, the molecular mechanism of TPTD is not fully elucidated yet. Therefore, future study focusing on the underlying mechanisms, including the effects of TPTD in vitro, is warranted. Currently, osteoporosis is the second leading cause for patients to be bedridden. It is believed that the number of patients using bone resorption suppression drugs, such as BP formulation, will increase further for preventing the deterioration of quality of life. However, the number of residual teeth in elderly people has increased owing to the progress of dental and medical technology. With the increase in number of residual teeth, the risk of infection due to caries and periodontal disease also increases, and consequently, the number of BRONJ patients will increase in the future. Therefore, establishment of TPTD therapeutic strategy for BRONJ is an urgent requirement, and we hope that this research would be helpful in establishing the ideal BRONJ therapy model by using TPTD adjunctive administration.

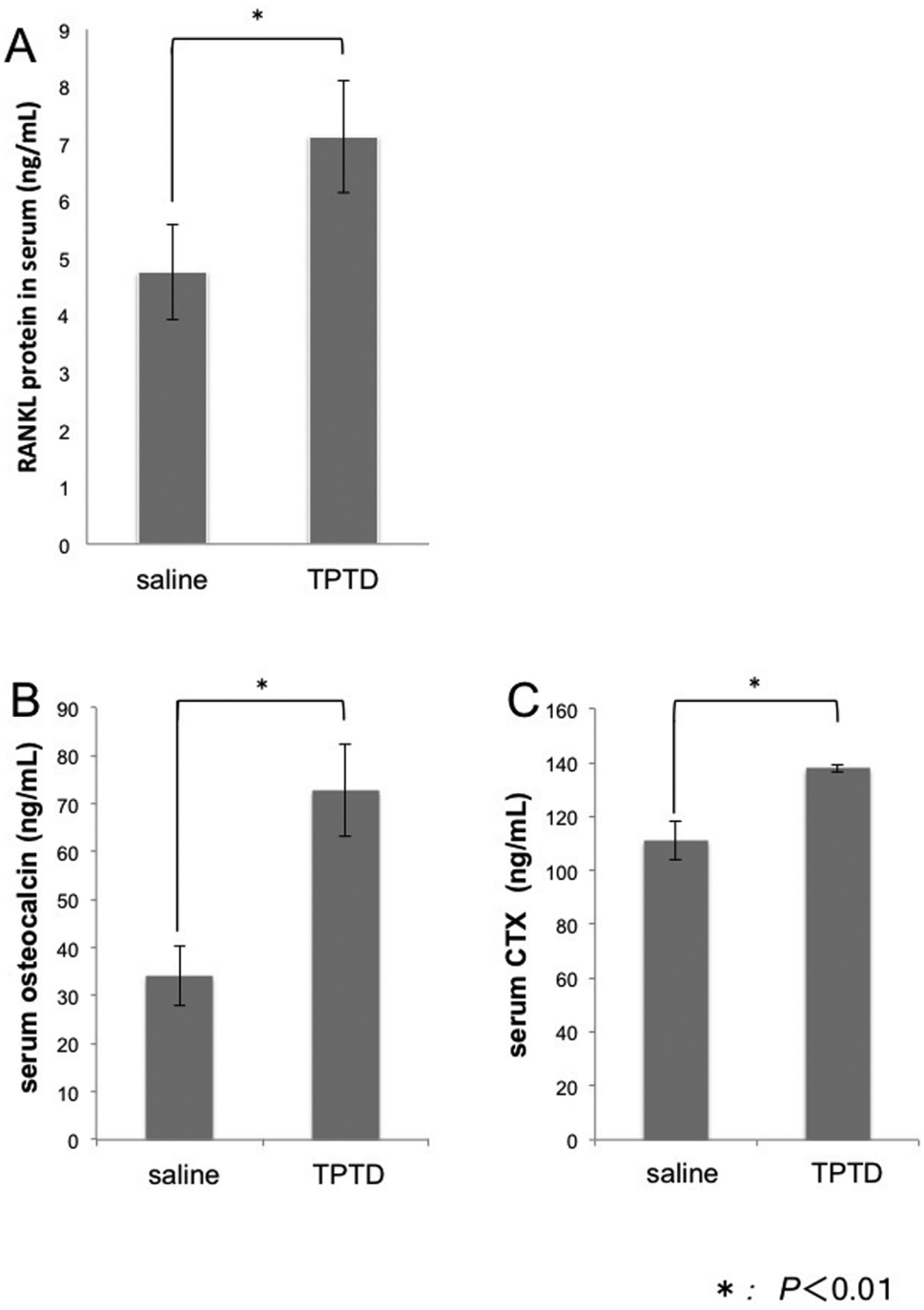

Fig. 7. RANKL protein level and bone marker (osteocalcin, CTX) levels detected by ELISA.
(A) The amount of RANKL protein significantly increased in the TPTD group compared to the saline group ($P < 0.01$). (B, C) We detected the levels of bone markers (osteocalcin, CTX) after 4 weeks of TPTD injection. The amount of osteocalcin and CTX protein levels significantly increased in the TPTD group compared to the saline group ($P < 0.01$).

Conflict of interest

All authors have no conflicts of interest.

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