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Local injection of RANKL facilitates tooth movement and alveolar bone remodelling

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Abstract

Objectives: To investigate the effect of local injection of receptor activator of nuclear factor kappa B ligand (RANKL) on experimental tooth movement and subsequent alveolar bone remodelling in mice.

Materials and Methods: Sixty mice were randomised to receive daily local RANKL or phosphate-buffered saline injections in the buccal premaxillary bone for 14 of 21 days of incisor movement, followed by a 21-day retention period. Five mice from each group were euthanised on days 0, 3, 7, 14, 21 and 42, and specimens were prepared for haematoxylin and eosin, tartrate-resistant acid phosphatase and immunohistochemical staining. Five mice from each group were subjected to serial microcomputed tomography until day 42 for tooth movement and bone volume quantification.

Results: The experimental group showed significantly greater tooth movement and bone volume reduction on days 14 and 21; an increased osteoclast number on days 3, 7, 14 and 21; and no difference on day 42. Higher RANKL expression was observed on days 7 and 14, with remarkable alkaline phosphatase activity. No significant systemic changes were observed.

Conclusion: Local RANKL injection leads to increased osteoclastic activity and facilitates tooth movement, followed by subsequent alveolar bone formation; this implies a reversible transitional acceleration of bone resorption.

KEYWORDS

bone remodelling, local injection, microcomputed tomography, receptor activator of nuclear factor kappa B ligand, tooth movement

1 | INTRODUCTION

Mechanical stress induces orthodontic tooth movement via alveolar bone remodelling, which involves balanced action of osteoclasts and osteoblasts (Zainal Ariffin, Yamamoto, Zainol Abidin, Megat Abdul Wahab, & Zainal Ariffin, 2011). Numerous mediators such as cytokines, growth factors and colony-stimulating factors are released from the periodontal ligament (PDL) and bone for osteoclastogenesis (Krishnan & Davidovitch, 2006). There are two factors, namely macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL), that are essential for osteoclastogenesis (Lacey et al., 1998). In particular, RANKL is a powerful stimulator in the entire osteoclastic differentiation process, leading to bone regeneration and remodelling (Boyce & Xing, 2008; Khosla, 2001). RANKL molecules bind to the RANK receptors on the surface of osteoclast lineage cells and promote osteoclast formation, function and survival. In contrast, osteoprotegerin (OPG) has been shown to counterbalance severe bone loss, playing a role as a decoy

receptor that competes with RANK for RANKL binding (Ozaki et al., 2017). Various experimental approaches using RANKL to facilitate bone resorption and turnover have been attempted. Transgenic mice overexpressing RANKL developed severe osteoporosis, followed by high bone turnover, low bone mineral density (BMD) and increased cortical porosity (Mizuno et al., 2002). During an experimental tooth movement involving the incisors in OPG knockout (OPG−/−) mice, osteoclasts were induced in periodontal tissues, RANKL and OPG in the periodontal tissues have been shown to be important determinants regulating balanced alveolar bone growth (Oshiro, Shiotani, Shibasaki, & Sasaki, 2002), implying tight regulation of bone resorption and remodelling in vivo, with one counteracting the other.

Intraperitoneal administration of soluble recombinant RANKL in mice significantly increased the bone turnover and endocortical bone resorption, and decreased the bone volume, mineralisation and strength (Lloyd et al., 2008; Yuan et al., 2008). However, systemic injection can cause systemic side effects, such as osteoporosis (Yasuda, 2013). On the other hand, local administration of RANKL may increase the activity of osteoclasts at the desired site without causing systemic side effects. Kanzaki et al. (2006) showed that transfer of the RANKL gene to periodontal tissue locally activated osteoclastogenesis and accelerated the rate of experimental tooth movement in rats. However, possible systemic side effects caused by virus injection have not been clarified, which may have been the reason why clinical application has been scarce. In order to increase the local concentration of RANKL, Chang et al. (2007) implanted collagen films with RANKL in mice calvarias for five days and compared the findings with control mice. They found that the relative bone volume decreased and the bone marrow space increased because of RANKL-stimulated bone resorption; however, the implantation procedure required surgical intervention, including incision and flap reflection. In this context, repeated injection of peptides has been proposed in a few studies (Li et al., 2015; Luo et al., 2016) because of its commercial availability and easier handling. Previous studies have investigated the different administration routes for RANKL. However, few studies have investigated the effects of local injection of RANKL on orthodontic tooth movement in mice. It is hypothesised that local injection of RANKL in the area of alveolar cortical bone can facilitate tooth movement and bone remodelling and minimise the side effects associated with other administration routes. The purposes of the present mouse model study were twofold; (a) to investigate the effects of local RANKL injection on experimental tooth movement and bone remodelling and (b) to observe bone healing during the retention period.

2 | MATERIALS AND METHODS

2.1 | Animals and administration

Sixty male, 6-week-old ICR mice with an average weight of 30 g were purchased from Koatech (Koatech, Pyeongtaek, Korea). The mice were fed powdered fodder to prevent the influence of masticatory forces on the incisors, and they were maintained for a week on a 12-hr light/dark cycle at a constant temperature of 23°C and relative humidity of 50% for acclimatisation in order to compensate for their different origins. The experiments of this study were approved by the institutional animal care and use committee of Yonsei University (approval number 2016-0081, 19 April 2016).

The mice were randomly divided into an experimental group and a control group. Mice in the experimental group received local injections of 0.04-μg/g (body weight) RANKL (Peprotech, Rocky Hill, NJ, USA) dissolved in 10-μl phosphate-buffered saline (PBS), while those in the control group received local injections of 10-μl PBS (Figure 1a). In both groups, injections were subperiosteally administered to the buccal premaxillary bone on each side using 0.5-ml insulin syringes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) under anaesthesia with 0.006-ml/10 g zolazepam (Zoletil 50, Virbac Lab Carros, France) and 0.004-ml/10 g xylazine (Rumpun, Bayer Korea Ltd, Korea). The first injection was administered on the day of orthodontic appliance insertion, followed by daily injections for 14 days.

2.2 | Tooth movement model

All mice except those euthanised on day 0 were fitted with the orthodontic appliance. The experimental appliance was a V-shaped helical loop comprising two turns of 0.012-inch stainless steel round wire (G&H, Franklin, IN, USA). The coil diameter was 1 mm, and the two arms of the spring were 10-mm long with an angle of 28° between them. A small hole was drilled directly below the gingival papilla between the maxillary central incisors using a 1/4 round bur. The appliance was subsequently inserted into the hole, and the ends

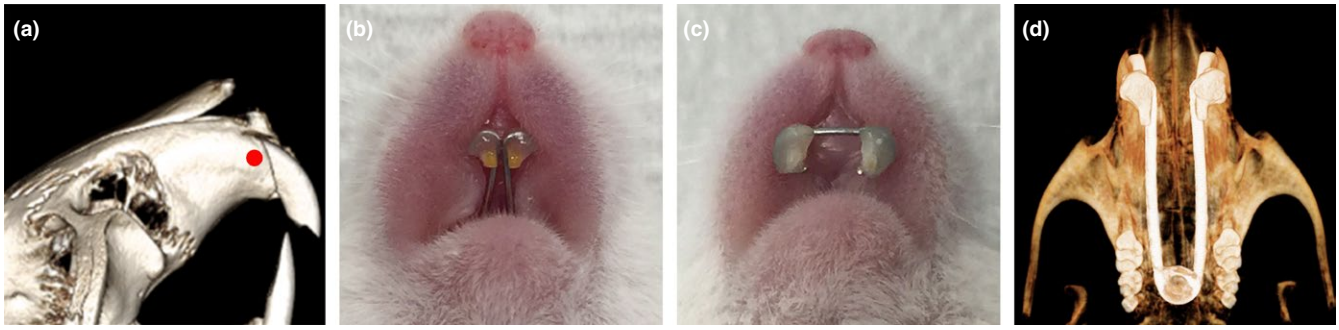

FIGURE 1 Local injection of RANKL or PBS and orthodontic tooth movement in mice. (a) Point of injection in the premaxillary bone (red dot). (b) Oral view of the tooth movement appliance. (c) Radiographic view of tooth movement. (d) Oral view of the retention appliance

of the helical spring arms were fixed to the incisors with composite resin (Figure 1b). Additionally, the plane, composed of two arms, was placed parallel to the palatal plane.

The helical spring was activated for buccal movement of the incisors by using an initial force of 35 g, which was proven to be an optimal force for orthodontic tooth movement in mice and exhibits no side effects (Taddei et al., 2012). There was no additional activity during the experimental tooth movement (Figure 1c). After euthanasia, the springs were checked to confirm whether the force was being continuously applied. The mandibular incisors were grinded every 3 days to eliminate occlusal interference with the appliance. After 21 days of tooth movement, the appliances were removed and replaced by retention appliances which made of 0.012-inch stainless steel round wire and were fixed to the incisors with composite resin to maintain the tooth position for the next 21 days (Figure 1d). There was no appliance dislodgement and no mice dead during the entire experiment period.

The entire experiment could be divided into the following three periods: day 0 to day 14, which was the period of tooth movement with daily RANKL or PBS injections; day 14 to day 21, which was the period of tooth movement without any injections; and day 21 to day 42, which was the retention period (Figure 2).

2.3 | Microcomputed tomography (micro-CT)

Five mice from each group were subjected to serial micro-CT scanning until day 42 for the quantification of orthodontic tooth movement and bone volume changes. The maxillae were subjected to in vivo micro-CT (Skyscan micro-CT 1076, Skyscan, Kontich, Belgium) under anaesthesia on days 0, 7, 14, 21 and 42. Scanning was performed using the following parameters: source voltage, 70 kV; source current, 139 μA; filter, 0.5 mm; resolution, 35 μm; exposure, 474 ms; and 360° rotation with a rotation step of 0.5°. The total scanning time for each specimen was 0.5 hr. The image data were exported in TIFF and converted to the DICOM format before import to an Invivo 5 program for the reconstruction of three-dimensional structures. Reconstructed images were used to measure the orthodontic tooth movement. For this measurement, a reference point and plane were required. The Mu and Bu landmarks were used in this study; these were derived from measurements used in a previous experimental study (Ramirez-Yanez, Smid, Young, & Waters, 2005). The maxillary structures on both sides were maximally overlapped in the three-dimensional image and sectioned by a line passing through the Mu and Bu points (Figure 3a). In that plane, the width between the incisors and the width of the inter-premaxillary suture were measured (Figure 3b). Because the appliance moves the tooth and separates the inter-premaxillary suture, the width of the inter-premaxillary suture was subtracted from the width between the incisors in order to overcome the effect of the suture split.

- Width between the incisors: distance between the mesiolabial line angles of the incisors
- Width of the inter-premaxillary suture: distance between mesiopalatal points on the alveolar bone crest on both sides
- Amount of tooth movement = (Width between the incisors − Width of the inter-premaxillary suture) − (Width between the incisors on day 0 − Width of the inter-premaxillary on day 0)

The micro-CT images were also used for the quantitative analyses of bone changes during the experimental period. The horizontal plane was established using the Mu and Bu points on both sides. On coronal sections, the region of interest (ROI) in the buccal premaxillary bone was defined as the red portion between the top and bottom lines of the incisors (Figure 3c). Sagittally, ROI included the buccal bone anterior to the premaxillary suture. ROIs for the premaxillary bone volume were marked for the quantitative estimation of bone volume changes; the bone volume was measured on both sides, and the average was considered for each sample.

The distal femoral metaphysis, which was 0.5–2.2 mm from the growth plate, of the five mice sacrificed on days 0, 14, 21 and 42 was scanned by micro-CT for the assessment of trabecular BMD.

2.4 | Histological examination

Five mice from each group were euthanised on days 0, 3, 7, 14, 21 and 42 of the experiment. The maxillae were dissected and fixed in 4% paraformaldehyde solution at 4°C for 24 hr, followed by decalcification with 10% ethylene diamine tetraacetic acid (EDTA; pH, 7.4) at 4°C for 3 weeks. The EDTA solution was replaced every 3 days. The samples were dehydrated using a graded ethanol series and embedded in paraffin. Fifteen 4-μm-thick sections were obtained from 0.5 mm below the line passing through the nasomaxillary point (Ulgen, Baran, Kaya, & Karadede, 1997), tangential to the incisors (Figure 4a). The sections were stained using haematoxylin and eosin (H&E; Figure 4b).

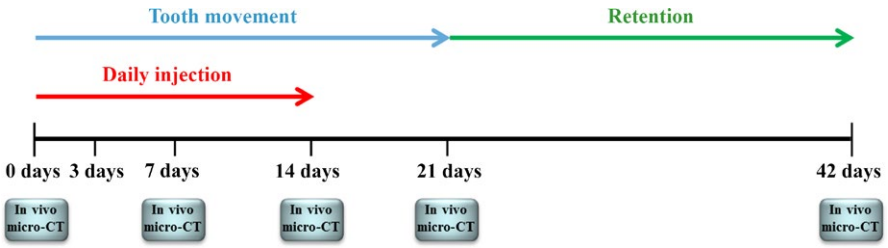

FIGURE 2 Schedule for experiment. Day 0 to day 14: tooth movement with daily RANKL or PBS injections; day 14 to day 21: tooth movement without any injections; day 21 to day 42: retention period; In vivo micro-CT scan on days 0, 7, 14, 21 and 42 [Colour figure can be viewed at wileyonlinelibrary.com]

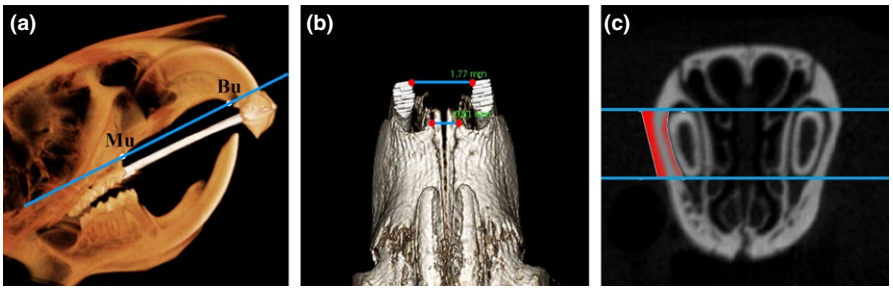

FIGURE 3 Measurement of the amount of tooth movement and bone volume on micro-CT images. (a) Section line (blue) passing through the Mu and Bu points. (b) Measurement of the width between the incisors and width of the inter-premaxillary suture. Width between the incisors: distance between the mesiolabial line angles of the incisors. Width of the inter-premaxillary suture: distance between mesiopalatal points on the alveolar bone crest on both sides. (c) Coronal section showing the region of interest in the premaxillary bone (red portion)

2.4.1 | Tartrate-resistant acid phosphatase (TRAP) staining

Osteoclasts were observed as TRAP-positive multinuclear cells on the alveolar bone surface using a TRAP staining kit (Sigma-Aldrich, St. Louis, MO, USA). The sections were dewaxed, rehydrated, incubated for 1 hr at 37°C in the TRAP reagent, which was prepared according to the manufacturer's instructions, and stained with haematoxylin. The number of TRAP-positive cells on both compression sides of the buccal bone were counted in three sections and averaged for each sample.

2.4.2 | Immunohistochemical staining

The tissue sections were placed in tris-buffered saline solution (TBS) after dewaxing in xylene and rehydration. Endogenous peroxidases were inhibited in methanol and hydrogen peroxide for 20 min at room temperature, followed by repeated washing in TBS. All sections were treated with 5% bovine serum albumin for 30 min to prevent non-specific background staining at room temperature. The sections were subsequently incubated overnight at 4°C with polyclonal goat anti-RANKL (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-alkaline phosphatase (ALP; 1:250, ab108337, Abcam, Cambridge, MA, USA) as primary antibodies. The slides were rinsed again and incubated for 30 min with a secondary antibody. Subsequently, the sections were developed with 3, 3′-diaminobenzidine chromogen and counterstained with haematoxylin.

2.4.3 | Interpretation of protein expression of RNAKL and ALP

Interpretation of protein expression was performed by using the weighted histoscore method (Witton, Hawe, Cooke, & Bartlett, 2004). The periodontal tissue cell intensity was scored as 0 (negative), 1 (light brown), 2 (brown) and 3 (dark brown). The final score was assessed as follows: score = (0 × percentage of negative cells) + (1 × percentage of light brown staining cells) + (2 × percentage of brown staining cells) + (3 × percentage of dark brown staining cells). Protein expression level was subdivided into low (histoscore: 0–100) and high (histoscore: 101–300) expressions, based on the histoscore. Five hot spots (areas that were strongly positive for RANKL or ALP) in each tissue section were selected, and the number of positive cells was counted out of 100 cells. Score was determined by the weighted histoscore method, and the average score of each sample was considered as the cut-off criterion for dividing between low and high expressions.

2.5 | Statistical analyses

Means and standard errors were calculated at each time point, and one-way analysis of variance (ANOVA) with the Tukey test was

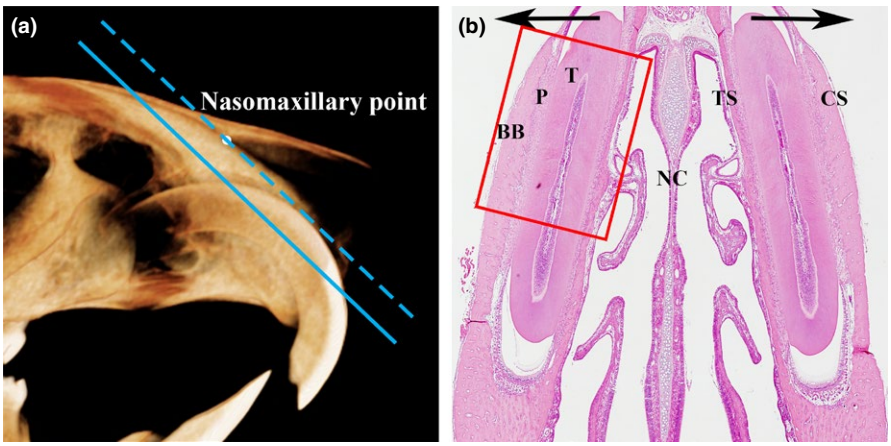

FIGURE 4 Paraffin section prepared after the sacrifice of mice. (a) Paraffin section plane. The blue-dotted line is passing through the nasomaxillary point, tangential to the incisors. The blue line is the paraffin section plane, which is 0.5 mm below the blue-dotted line. (b) Paraffin section stained with haematoxylin and eosin. The black arrows indicate the direction of force, and the red box indicates the region of observation. BB: buccal bone; CS: compression side; NC: nasal cavity; P: periodontal ligament; T: tooth; TS: tension side

performed for intergroup comparisons during the follow-up periods. Differences between the two groups at each time point were evaluated using an unpaired *t* test. Differences in protein expression between groups were assessed by the chi-squared test. The level of significance was 95% for all tests.

3 | RESULTS

3.1 | Animals

The body weight of the mice gradually decreased during the tooth movement period and recovered during the retention period, with no significant differences between the control and experimental groups throughout the experiment (Figure 5a).

Local RANKL injection did not affect BMD in the distal femoral metaphysis, with no significant differences between the two groups on days 0, 14, 21 and 42 (Figure 5b).

3.2 | Effect of local RANKL injection on root resorption, experimental tooth movement and bone volume

Root resorption was not observed in the control group, except in a single case in the experimental group on day 21, as assessed in tissue sections.

There was no significant difference in the amount of tooth movement between the control (0.52 ± 0.06 mm) and experimental (0.49 ± 0.07 mm) groups on day 7 (Figure 5c). However, there were significant differences between groups on days 14 (control group 0.79 ± 0.12 mm, experimental group 1.15 ± 0.27 mm, $p < 0.05$) and 21 (control group 1.07 ± 0.12 mm, experimental group 1.55 ± 0.22 mm, $p < 0.01$). In addition, the amount of separation of premaxillary suture between groups exhibited no significant differences during tooth movement.

Although the bone volume decreased in both groups, the experimental group showed significantly lower volume than the control group only on day 14 (control group 1.03 ± 0.10 mm^3, experimental group 0.88 ± 0.08 mm^3, $p < 0.05$); there were no significant differences between the two groups on days 21 and 42 (Figure 5d).

3.3 | Effect of local RANKL injection on bone remodelling

Figure 6a shows the micro-CT and three-dimensional reconstruction findings for the same area in live mice from both groups at each time point. Bone resorption occurred in both groups during the experimental tooth movement period. Compared with the control group, the experimental group showed significant bone resorption on day 14.

Periodontal tissue changes were observed in the tissue sections stained with H&E. For the duration of tooth movement, buccal bone on the compression side was clearly reduced in both groups. Bone resorption in the experimental group was more obvious than in the control group on days 14 and 21 (Figure 6b).

3.4 | Effect of local RANKL injection on the number of osteoclasts

TRAP-positive osteoclasts were found along the bone surface on the compression side, and this accelerated the resorption of the buccal bone, leading to the formation of an irregular bone surface (Figure 7a–d). The number of osteoclasts was noted to gradually increase in both groups (Figure 7e). There was significantly greater number of TRAP-positive osteoclasts in the experimental group than in the control group on days 3, 7, 14 and 21. In both groups, the number of osteoclasts significantly increased on days 14 and 21 (Figure 7f).

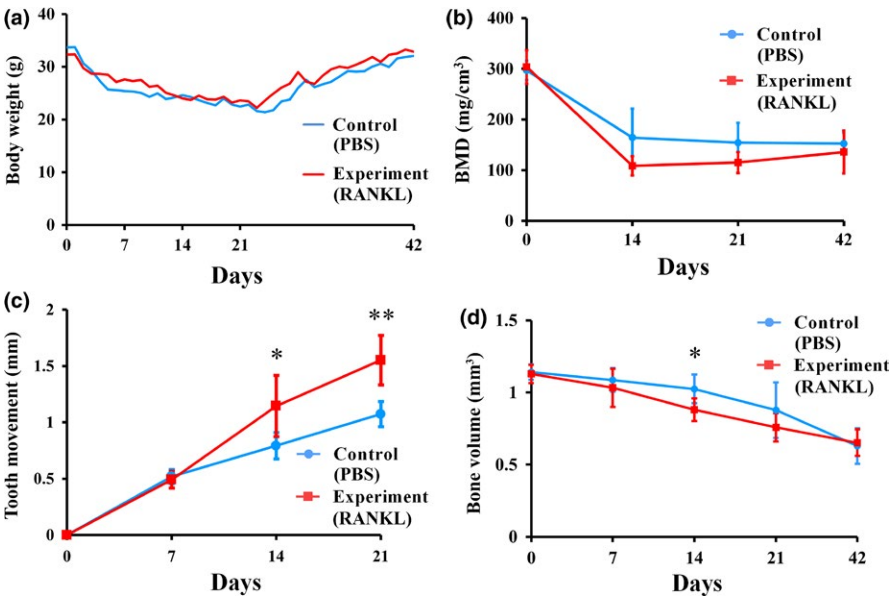

FIGURE 5 Effects of local injection of RANKL in mice. (a) The body weight shows a decrease during the tooth movement period (days 0–21), followed by recovery during the retention period (days 21–42). (b) The BMD in the distal femoral metaphysis shows no significant difference between the control (PBS injection) and experimental (RANKL injection) groups on days 0, 14, 21 and 42. (c) Amount of tooth movement over time. The amount of tooth movement is significantly greater in the experimental group than in the control group ($n = 5$) $^{*}p < 0.05$, $^{**}p < 0.01$. (d) Time course of changes in the bone volume. There is a significant difference between the two groups only on day 14, with no differences on days 21 and 42 ($n = 5$) $^{*}p < 0.05$

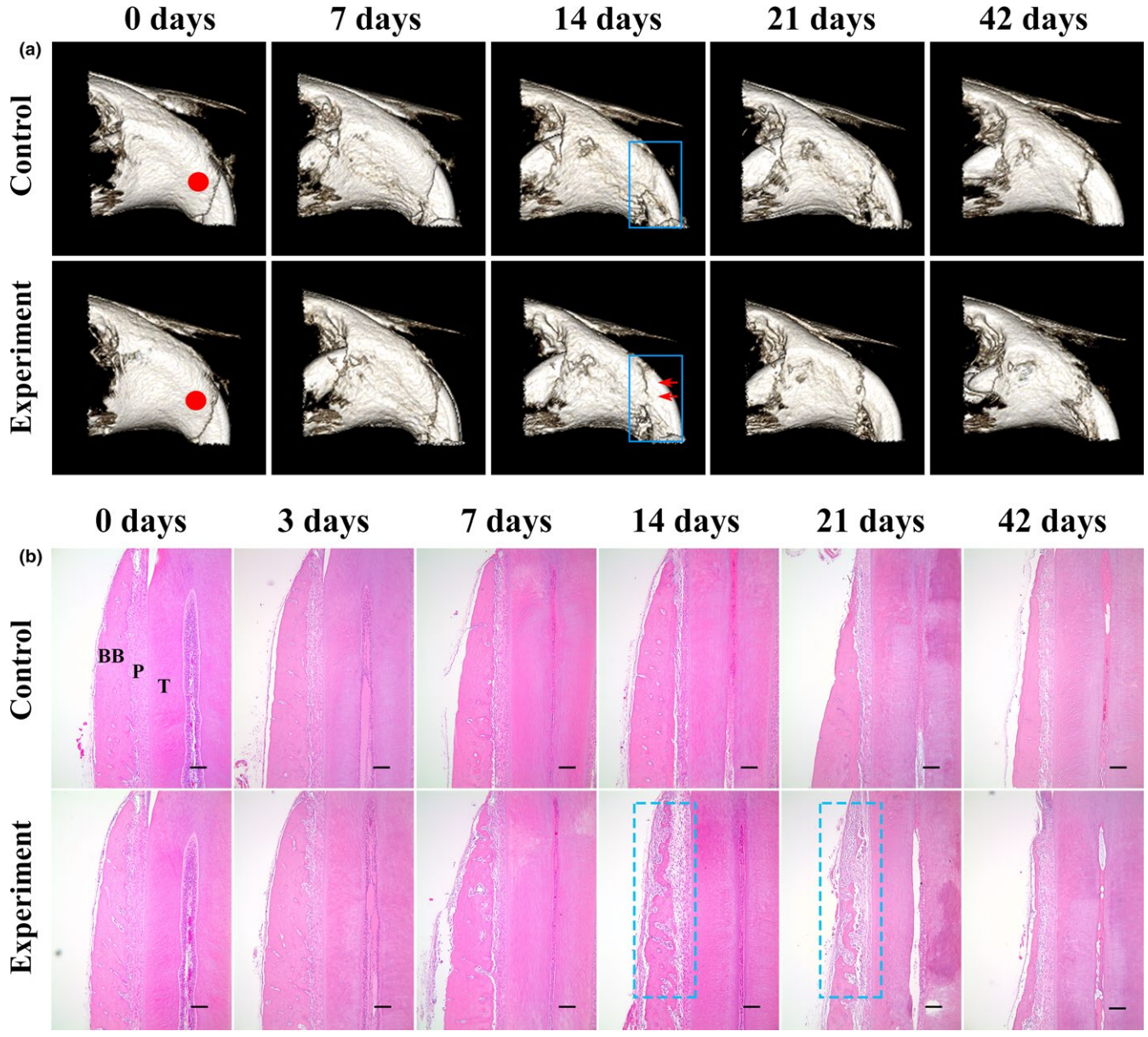

FIGURE 6 Effects of local injection of RANKL on bone remodelling in mice. (a) Microcomputed tomography and three-dimensional reconstructed images of the maxillary bone at each time point. Compared with the control group (PBS injection), the experimental group (RANKL injection) shows significant bone volume reduction on day 14. Red dot: injection point, red arrows: region of premaxillary bone resorption. (b) Histological changes in the tissues on the compression side. Haematoxylin and eosin-stained paraffin sections on days 0, 3, 7, 14, 21 and 42. The blue-dotted box shows significant bone volume reduction on days 14 and 21 in the experimental group. BB: buccal bone; P: periodontal ligament; T: tooth (×100 magnification). Scale bar = 100 μm

3.5 | RANKL and ALP expression in the periodontal tissue

Receptor activator of nuclear factor kappa B ligand expression was observed in the periodontal tissue on the compression side during the tooth movement period. There were few RANKL-positive cells on day 7 in the control group. However, strong RANKL expression was observed on days 7 ($p < 0.05$) and 14 ($p < 0.05$) in the experimental group (Figure 8a).

Anti-alkaline phosphatase was detected in osteoblasts and periodontal ligaments adjacent to the alveolar bone. ALP expression was significantly higher on the tension side than on the compression side during the tooth movement period. In addition, on day 21 ($p < 0.05$), the experimental group showed stronger expression on the compression side, compared with the control group (Figure 8b).

4 | DISCUSSION

In clinical applications of various agents, evaluations of both pharmacological effects and subsequent recovery outcomes are necessary to determine overall safety. In this study, we used a mouse model

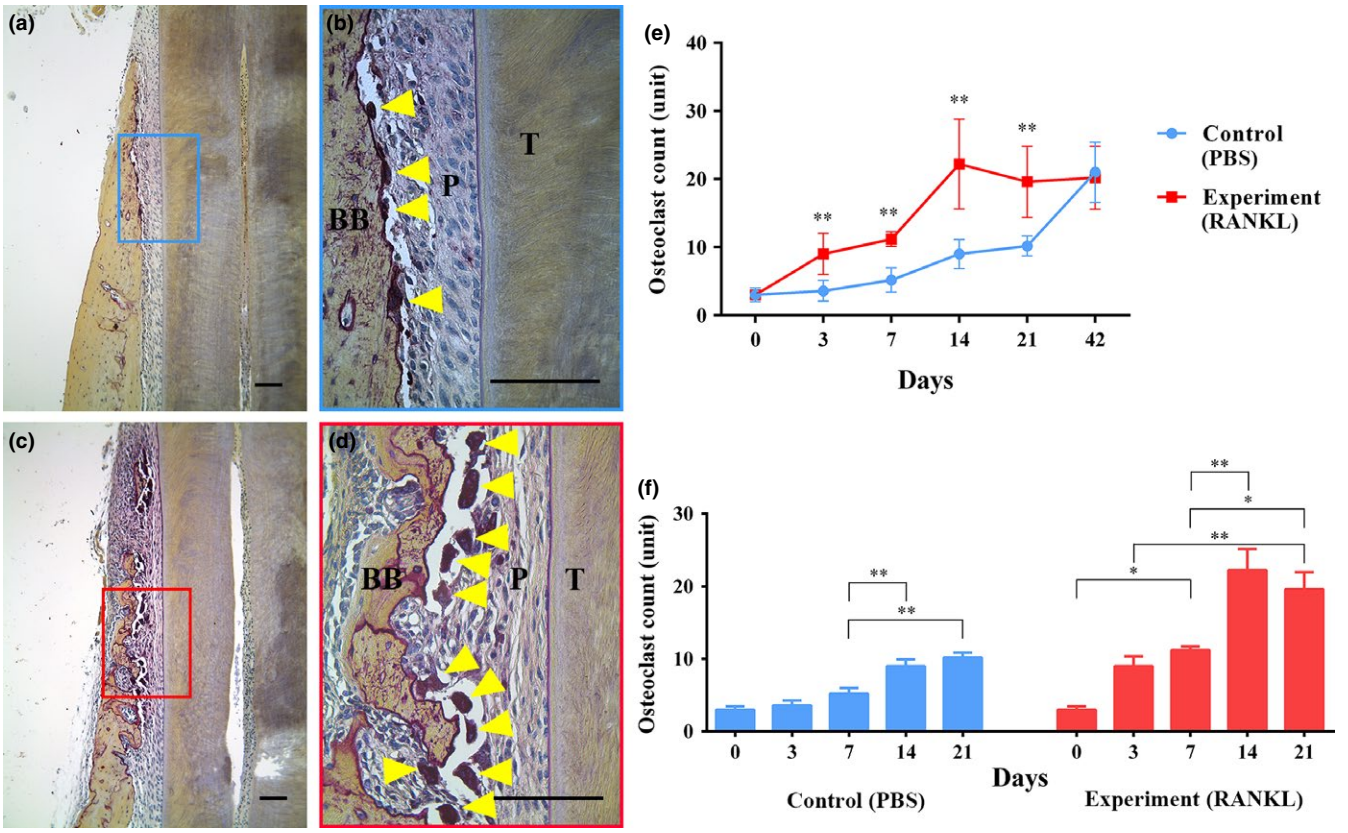

FIGURE 7 Effects of local injection of RANKL on the osteoclast number in mice. (a) TRAP staining of a section obtained from the control group (PBS injection) on day 21. (b) Enlarged image of the blue box in (a). (c) TRAP staining of a section obtained from the experimental group (RANKL injection) on day 21. (d) Enlarged image of the blue box in (c). The yellow arrowheads indicate osteoclasts. T: tooth, P: periodontal ligament, BB: buccal bone. a, c: ×100 magnification b, d: ×400 magnification. Scale bar = 100 μm. (e) Intergroup comparison of the number of TRAP-positive osteoclasts during the experimental period. The number of osteoclasts is significantly larger in the experimental group than in the control group (n = 5). *p < 0.05, **p < 0.01. (f) Intragroup comparison of the number of TRAP-positive osteoclasts during the 21-day tooth movement period. *p < 0.05, **p < 0.01

to investigate the effect of local RANKL injection on experimental tooth movement and to observe the bone remodelling pattern during both the tooth movement and retention phase in mice.

In previous studies, the majority of experimental orthodontic tooth movement models involved mesial traction of the maxillary first molar, using the incisors as anchorage; first molar displacement was measured to evaluate the amount of tooth movement. In practice, however, most orthodontic tooth movement involves incisor displacement and tooth movement "against" the cortical plate (e.g., lateral displacement or lingual retraction); this tends to be a rate-limiting step of treatment (Handelman, 1996). Moreover, in rodents, the molars tend to drift distally, and the measurement of tooth movement may be inaccurate (Ren, Maltha, & Kuijpers-Jagtman, 2004). The main goal in this study was to observe the bone response to locally injected RANKL, for which a cortical plate covering the majority of root surface was necessary. Local injection of RANKL was presumed to initiate bone resorption only on the superficial area, according to previous reports (Li et al., 2015; Luo et al., 2016). Therefore, the molar protraction model was not considered suitable as an experimental model for our study of the cortical bone response. In order to meet these two conditions, the target root must be covered by a cortical plate. Therefore, in the present study, the incisors of mice were moved towards the buccal plate by using a helical spring in order to achieve experimental tooth movement. Similar to the calvaria, the lateral cortical plate is a thin cortex surrounding the tooth and was considered suitable for observing changes on both the periodontal and periosteal sides. However, in order to overcome the effect of premaxillary suture separation, the gap in the palatal suture was subtracted from the interincisor width. In order to eliminate the effect of constant incisor eruption in mice, the mandibular incisors were grinded and the vertical position of the wire hole was traced. There was no significant difference in the length of gingival papilla-to-wire hole between the two groups (data not shown).

In order to observe the changes in bone morphology, we used a serial in vivo (whole mount) micro-CT device to obtain images for live mice. The use of in vivo micro-CT facilitates longitudinal follow-up in the same animal, is effective in minimising individual variations, and accurately measures local architectural changes in bone over time (Bouxsein et al., 2010). For stabilisation of the animal during micro-CT, the mice were fixed on the bed and anaesthetised to minimise movement. ROIs in the premaxillary bone were marked and

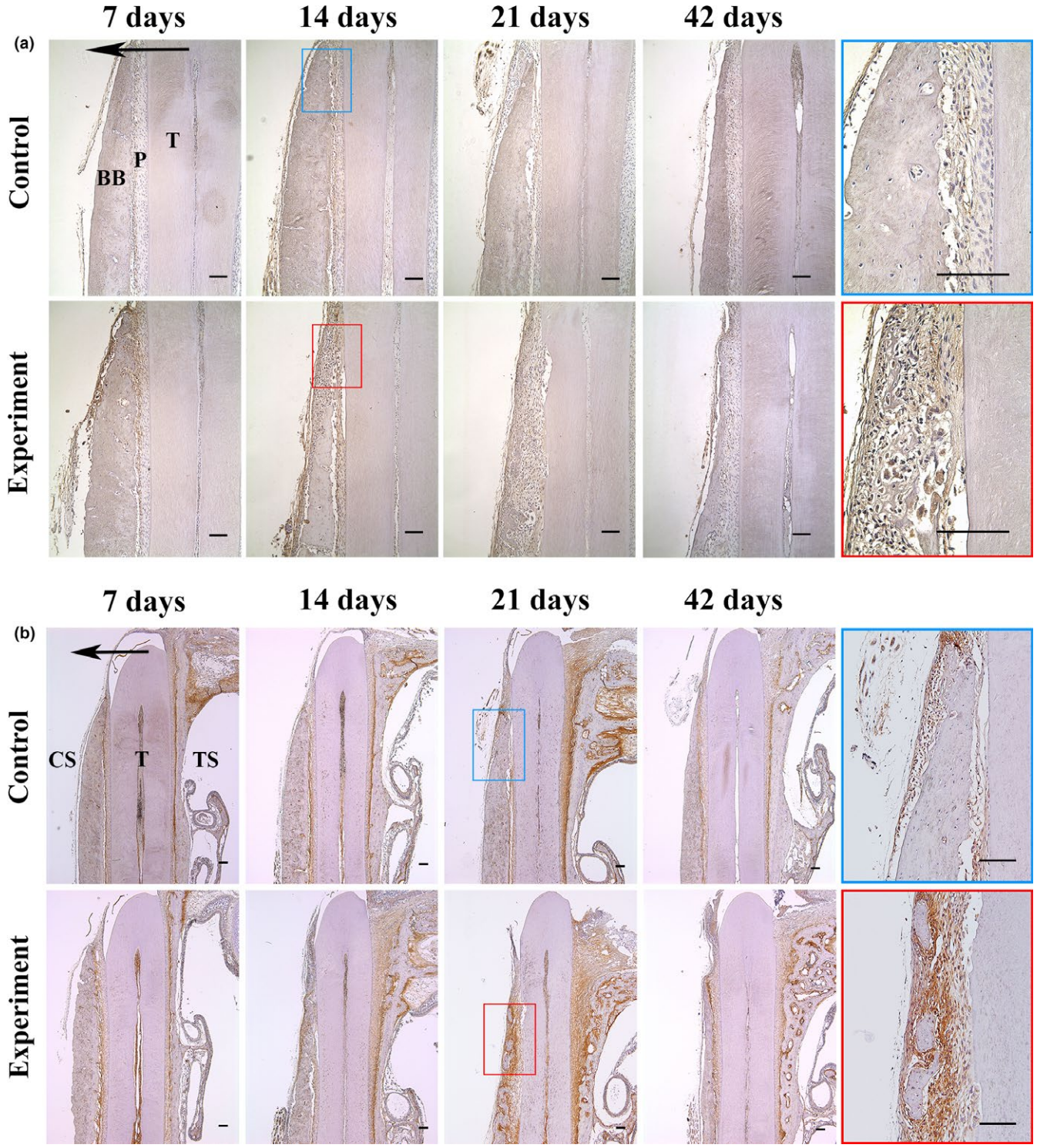

FIGURE 8 Expression of RANKL and ALP in the periodontal tissue of mice that received local RANKL (experimental group) or PBS (control group) injections during the experimental period. (a) RANKL expressions on the compression side during the experimental period. RANKL expression is significantly higher on days 7 ($p < 0.05$) and 14 ($p < 0.05$) in the experimental group than in the control group (×100 magnification). The Figures with blue and red borders are enlarged images of the boxes in the 14-day images for the control and experimental groups, respectively (×400 magnification). BB: buccal bone; P: periodontal ligament; T: tooth. Scale bar = 100 μm. (b) ALP expression in the periodontal tissue during the experimental period. ALP expression is significantly higher on the tension side than on the compression side during the 21-day tooth movement period. On day 21 ($p < 0.05$), the expression on the compression side is higher in the experimental group than in the control group (×50 magnification). The Figures with blue and red borders are enlarged images of the boxes in the 21-day images for the control and experimental groups, respectively (×200 magnification). Scale bar = 100 μm

evaluated for the quantification of bone volume changes during the experimental period.

In soluble RANKL-injected mice, Tomimori et al. (Tomimori et al., 2009) found that the serum levels of exogenous soluble RANKL peaked at 4 hr after injection and decreased rapidly thereafter, becoming undetectable at 24 hr. A previous study also proposed repeated injections for 2 weeks in order to maintain the local concentration of RANKL (Li et al., 2015). Accordingly, in the present study, RANKL was injected daily into the premaxillary bone for 14 days in order to maintain its local concentration. The body weight of the mice was observed to decrease gradually, possibly because the incisal contacts were eliminated, and the mice could not eat well during the tooth movement period. However, there were no significant differences in body weight between the two groups at any point during the study. In addition, there was no difference in BMD in the distal femoral metaphysis between the two groups after 14 days of daily RANKL injections and at the end of the experiment (day 42). Therefore, we can infer that daily local injections of RANKL did not show remarkable systemic effects.

Shiotani et al. (Shiotani, Shibasaki, & Sasaki, 2001) have shown the presence of RANKL in periodontal tissues during experimental tooth movement. RANKL produced by periodontal ligament and bone lining cells provide the major driving force for tooth movement and osteoclastogenesis in response to orthodontic forces (Yang et al., 2018). Local RANKL injection can recruit peripheral monocytes and macrophages (Jin, Li, & Yu, 2011) and has the ability to produce inflammatory cytokines such as interleukin (IL)-1b, IL-6, IL-11 and tumour necrosis factor-alpha (TNFα), which probably mediate bone remodelling by stimulating RANKL expression during orthodontic tooth movement (Kohli & Kohli, 2011; Saito, Saito, Ngan, Shanfeld, & Davidovitch, 1991). Yamaguchi and Kasai (2005) suggested that RANKL was regulated by inflammatory cytokines in the periodontal ligament in response to mechanical stress. In the present study, RANKL expression on days 7 and 14 was higher in the experimental group than in the control group, which implied an increased local concentration of RANKL caused by repeated daily injections (Figure 8a). However, we do not know whether the observed RANKL was intrinsic or extrinsic, which a limitation of this study.

ALP is a well-known marker of bone formation (Christenson, 1997). Because of the combined pharmacological and mechanical stimuli applied to the cortical bone, it was crucial to determine whether the bone resorption is irreversible. In the present study, the tension side showed significantly higher ALP expression than did the compression side during the tooth movement period, which is relevant to the conventional pressure and tension theory of tooth movement. However, on day 21, the compression side showed a larger ALP-positive area in the experimental group than in the control group, indicating significant osteogenesis in the resorbed area. Therefore, we can infer that an increased local concentration is associated with bone resorption as well as subsequent bone formation, which implies spontaneous recovery in vivo.

With regard to the amount of tooth movement, significantly greater movement was observed in the RANKL group than in the control group, which is consistent with the above findings. Specifically, acceleration of tooth movement was observed after a week, with a pattern different from that in previous studies showing immediate acceleration followed by a normal speed of tooth movement (Iino et al., 2007; Ren, Maltha, Van 't Hof, & Kuijpers-Jagtman, 2003). This is presumably because of the presence of a lag phase in both groups. Considering the timing of osteoclast appearance and thinning of the cortical bone, a relative delay in tooth movement is considered reasonable. The experimental group showed significantly lower bone volume than the control group on day 14, thinning of the cortical bone results in a decrease of resistance that prevents tooth movement. Due to this reason, although there were no injections of RANKL from days 14 to 21, the amount of tooth movement was more increased on day 21 compared with day 14.

RANKL in periodontal ligament cells contributes to alveolar remodelling and to root resorption during orthodontic tooth movement by up-regulation of osteoclastogenesis (Tyrovola, Spyropoulos, Makou, & Perrea, 2008). In this study, although significantly greater movement was observed in the experimental group, root resorption occurred only in a single case, on the compression side, in the experimental group on day 21. Therefore, we inferred that daily local injections of RANKL did not show remarkable effects on external apical root resorption caused by orthodontic tooth movement.

Kanzaki et al. (2006) reported an increase in the number of osteoclasts following local RANKL gene transfer with or without orthodontic force; the number was high on day 3 and decreased thereafter in the group with no tooth movement. In the present study, the number of osteoclasts continuously increased in both the control and experimental groups from days 0 to 14, with a significantly greater increase in the experimental group up to 21 days. In the experimental group, the number of osteoclasts was smaller on day 21 than on day 14, probably because there was no additional injection of RANKL during this period. Consequently, it was considered that RANKL only causes a temporary increase in osteoclastogenesis. During the retention period, however, the control group showed a constant increase in the osteoclast number, resulting in the absence of significant differences between the two groups. Histological analysis showed that osteoclasts were observed in the anterior aspect of the premaxillary bone on the compression side during the tooth movement period. This finding was different in the retention period, during which overall distribution was observed on the compression side of the buccal bone, with continuous bone remodelling. The alveolar bone of animals is dense with relatively few marrow spaces, and anatomical details may influence the resorption process on the compression side in experiments of a longer duration (Reitan & Kvam, 1971). Notably, although the moved teeth were fixed, the maxillary buccal bone was under continuous compression because of relapse of the separated inter-premaxillary suture. This may be the reason for the increase of osteoclasts during the retention period in the present study. In terms of bone volume, the temporal decrease in the experimental group was recovered at the end of retention period. In both groups, however, the absolute bone volume was reduced as a result of

orthodontic tooth movement. Taken together, local RANKL injection may facilitate reversible bone resorption and tooth movement that can be recovered over time.

With regard to clinical implications, an increase in the osteoclastic activity by local injection of RANKL in appropriate parts of the cortical bone can accelerate tooth movement; this suggests a practical application of RANKL without concerns about systemic or irreversible side effects. More advanced applications may include displacement of ankylosed teeth and/or release of a fused suture for orthopaedic correction. The limitations of this study included a short observation period for bone formation during the retention phase and the lack of dose-effectiveness because of the high cost of the reagent. Therefore, further studies are needed to reveal the various pharmacological effects of local RANKL injection.

5 | CONCLUSION

Repeated local injection of RANKL during orthodontic tooth movement can lead to increased osteoclastic activity on the periodontal side and accelerated tooth movement, followed by increased ALP activity in the entire cortical plate, indicating bone formation. Therefore, local injection may be a practical application of RANKL without concerns about systemic or irreversible side effects.

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTION

The study was designed by Kee-Joon Lee. Choo-ryung Chung and Chung-ju Hwang contributed to the improvement of study implementation. Chengri Li had contribution in the data collection and analysis and drafted the paper. All authors contributed to editing and critically revising the final manuscript.

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