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Original article

Does local injection of reveromycin A inhibit tooth movement without causing systemic side effects?

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Summary

Objective: To determine the feasibility of local inhibition of osteoclast activity and control of tooth movement with local intraoral reveromycin A (RMA) injection in model mice for experimental tooth movement.

Materials and methods: Eight-week-old wild-type mice (n = 6 per group) were divided into four groups consisting of two non-RMA groups that received normal saline for 14 (14-day non-RMA group) or 21 consecutive days (21-day non-RMA group) and 2 RMA groups that received RMA (1.0 mg/kg of weight) for 14 (14-day RMA group) or 21 consecutive days (21-day RMA group). RMA was injected locally into the buccal mucosa of the left first maxillary molar twice daily starting 3 days before placement of the 10-gf Ni-Ti closed coil spring. Tooth movement distance was analysed using micro-computed tomography. The effects on surrounding alveolar bone were evaluated by measuring the ratio of bone surface area to tissue surface area with haematoxylin-eosin-stained sections and counting the number of osteoclasts in periodontal tissue with TRAP-stained sections. Blood tests were performed and bone volume and trabecular separation at the tibial neck were measured to analyse systemic side effects.

Results: Local RMA injection inhibited tooth movement by 40.6 per cent, promoted alveolar bone volume maintenance by 37.4 per cent, and inhibited osteoclast activity around the tooth root at 21 days by 40.8 per cent. Systemic effects on osteoclasts or osteoblasts were not observed.

Conclusion: Local injection of RMA enabled control of tooth movement without systemic side effects in a mouse model.

Introduction

In orthodontic treatment, movement of target teeth often causes movement of anchor teeth as well. Therefore, the anchor teeth must be stabilized to ensure favourable therapeutic results. Conventionally, extraoral anchorage appliances, such as headgear, have been used to stabilize the anchor teeth, but their success depends greatly on patient cooperation. Recently, orthodontic anchor screws developed to secure anchor teeth for orthodontic treatment are frequently being used to ensure absolute anchorage (1–4). However, orthodontic anchor screws have the disadvantage of surgical invasiveness and of

potentially falling out (5, 6). Therefore, recent studies have investigated the control of tooth movement via pharmacological suppression or enhancement of bone metabolism (7–9).

When mechanical stress is applied to the teeth by orthodontic treatment, local and selective modelling and remodelling occurs through increased bone resorption (primarily mediated by osteoclasts) in alveolar bone on the pressure side, with concomitantly increased bone formation and growth (primarily mediated by osteoblasts) in alveolar bone on the tension side (10). Osteoclasts, which are important in the maintenance of bone homeostasis, are the only cells that break down and resorb calcified bone tissue. Osteoclast differentiation, maturation, and function are strictly regulated by receptor activator of NF-κB ligand (RANKL) expressed on the cell membrane of osteoclasts and bone marrow stromal cells (11). Osteoclasts and their progenitor cells recognize RANKL and differentiate into mature osteoclasts.

Reveromycin A (RMA) was recently identified as a selective inhibitor of osteoclast activity (12). RMA, an acidic polypeptide compound originally discovered in culture medium of actinomycetes (12), is typically not readily taken up by cells. However, it is selectively taken up by activated osteoclasts in an acidic environment because these cells secrete acid to dissolve bone (13). Uptake is not observed in osteoclast progenitor cells, but marked uptake is observed in activated osteoclasts with bone resorbing ability. In these cells, RMA inhibits the activity of its target molecule, isoleucyl tRNA synthetase, which inhibits protein synthesis and causes apoptosis, which then inhibits bone resorption (14). It also has a shorter half-life than existing bone resorption inhibitors (14).

In a previous study, we used an osteoprotegerin (OPG)-deficient mouse model of high-turnover osteoporosis to create model mice for experimental tooth movement, and discovered that tooth movement in these mice can be inhibited by intraperitoneal injection of RMA (15, 16). However, intraperitoneal RMA injection might also inhibit osteoclast activity in non-target tissues because RMA enters the systemic circulation. Lloyd *et al.* found that intraperitoneal injection of the osteoclast differentiation factor RANKL significantly increased bone turnover and resorption of cortical bone and reduced bone volume, calcification, and strength in mice (17), but might have caused systemic side effects resembling osteoporosis (18). However, local intraoral injection of RANKL in experimental tooth movement mice increased local osteoclast activity and increased the rate of tooth movement without causing systemic side effects (19). If local injection of RMA in mice with normal bone metabolism can suppress tooth movement without systemic side effects, in clinically, it may be an effective pharmacological approach with no systemic side effects for controlling tooth movement in orthodontic treatment.Therefore, to determine the optimal use of RMA clinically, we conducted a longer study to establish the possibility of local inhibition of osteoclast activity and control of tooth movement with local intraoral RMA injection in experimental tooth movement model mice and to assess the systemic effects of local injection.

Materials and methods

Animals and drug administration

This study used a total of 24 eight-week-old male wild-type (WT) C57BL/6J mice (n = 6 per group). The mice were purchased from CLEA Japan (Tokyo, Japan) and reared at the animal laboratory at Aichi Gakuin University School of Dentistry. Housing conditions were standardized at 22 ± 2°C and 50 ± 10 per cent humidity with lights on a 12-hour cycle. The mice were given free access to CE-2 powdered diet (CLEA Japan, Tokyo, Japan) and tap water. Laboratory animal management and research methods were approved by the Animal Care and Use Committee of Aichi Gakuin University School of Dentistry (Approval No. AGUD359) and were in accordance with guidelines for research on laboratory animals. The drug used in the experiments was RMA (reveromycin A 3Na salt). The mouse model of experimental tooth movement was created by intraperitoneal injection of a triple-anaesthesia cocktail of medetomidine hydrochloride (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), midazolam (Astellas Pharma, Inc., Tokyo, Japan), and butorphanol tartrate (Meiji Seika Pharma Co., Ltd.) (20) followed by placement of a 10-gf Ni–Ti closed coil spring between the maxillary incisor and left maxillary first molar to produce mesial movement for 14 or 21 days. These left teeth were evaluated as the experimental (loaded) group. The same procedure was carried out at the right first molar, and these teeth were evaluated as the control (unloaded) group (Figure 1a and 1b).

RMA injection

Eight-week-old WT mice (n = 6 per group) were divided into four groups consisting of two non-RMA groups that received normal saline for 14 (14-day non-RMA group) or 21 consecutive days (21-day non-RMA group) and 2 RMA groups that received RMA (1.0 mg/kg body weight) for 14 days (14-day RMA group) or 21 consecutive days (21-day RMA group). RMA was injected locally into the buccal mucosa of the left first maxillary molar twice daily starting at 3 days before placement of the 10-gf Ni–Ti closed coil spring (Figure 1a). Local injection of RMA was not performed

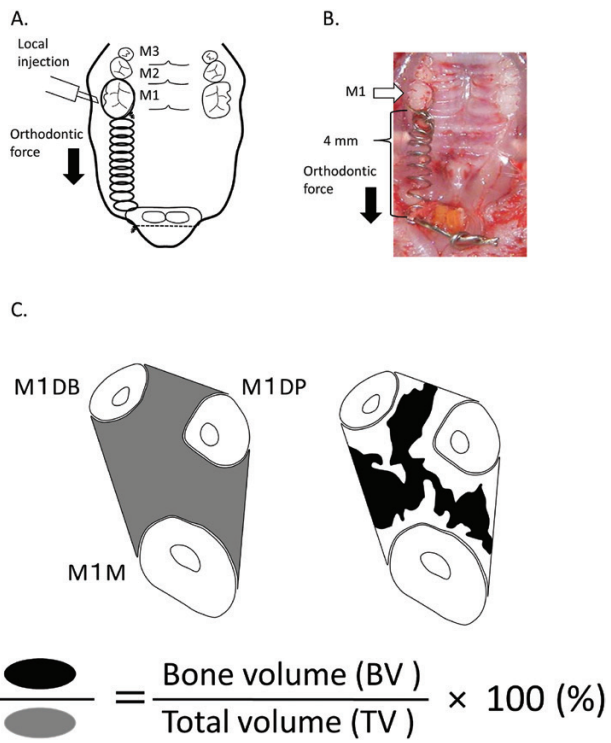

Figure 1. (a) Schematic diagram showing positioning of the closed coil, direction of experimental tooth movement, and site of local injection. (b) Placement of the closed coil at the first molar. (c) Sites of bone density measurement. M1DB, M1 distobuccal root; M1DP, M1 distopalatal root; M1M, M1 mesial root.

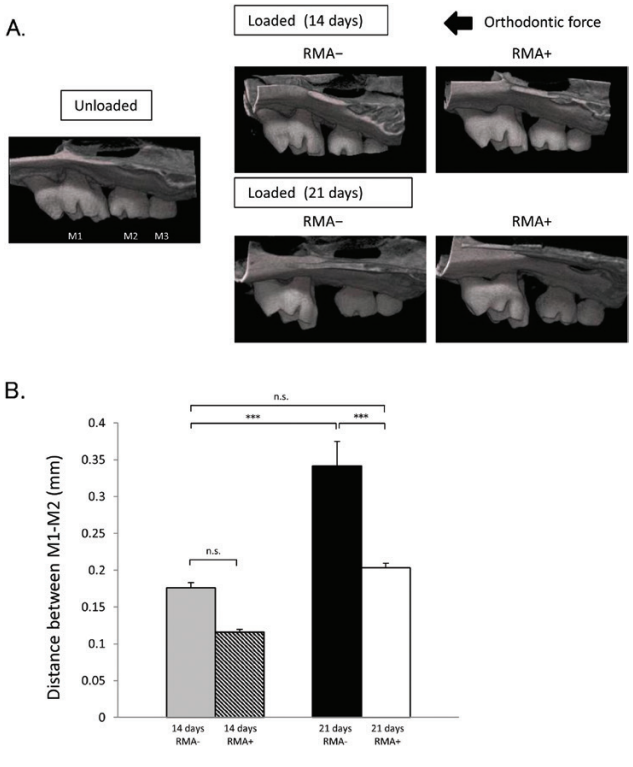

Figure 2. (a) Micro-CT images of the left first molar acquired after 14 and 21 days of experimental tooth movement. RMA+, RMA administration group; RMA–, saline administration group. (b) Distance between M1 and M2 (mm). n.s., not significant; ***, $P < 0.001$. RMA+, RMA administration group; RMA–, saline administration group.

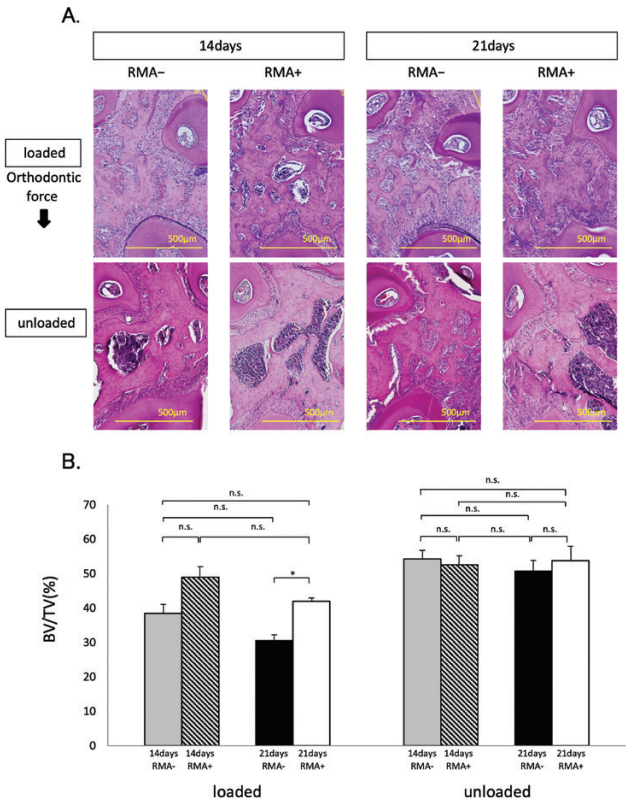

Figure 3 (a) Hematoxylin-eosin staining of periodontal tissue after 14 and 21 days of experimental tooth movement (alveolar bone around M1 root). (b) Representative bone volume (BV/TV).n.s., not significant; *, $P < 0.05$. RMA+, RMA administration group; RMA–, saline administration group.

under anaesthesia. The animals in the control group were subjected to the same stress by local injection of normal saline. Methods described by Tanaka *et al.* were referenced for RMA concentration and pre-dosing duration (15).

Micro-computed tomography

The maxillary bone and right tibial neck were harvested after 14 or 21 days of experimental tooth movement and evaluated with micro-computed tomography (CT; Rigaku, Tokyo, Japan). Tube voltage was 90 kV, tube current 88 μA, scanning time 2 minutes, and pixel size 20 × 20 × 20 μm. Tooth movement distance was analysed using TRI/3D-BON software (Ratoc System Engineering Co., Ltd., Tokyo, Japan). Maxillary bone movement was measured by adjusting the view, so the narrowest portion could be observed in sagittal and horizontal cross sections. For scans of the right tibial neck, 100 consecutive 50-μm thick slices were acquired. Cortical bone contours were mapped semi-automatically, and once removed, the ratio of bone surface area to tissue surface area (BV/TV) and trabecular separation (Tb.Sp) were calculated.

Histomorphometric evaluation

Maxillary bone was removed after 14 or 21 days of experimental tooth movement and fixed in 10 per cent neutral buffered formalin solution. Next, it was demineralized in 10 per cent EDTA (pH 7.2) for about 4 weeks at 4°C, embedded in paraffin following conventional methods, and cut into 5-μm consecutive horizontal tissue sections. The tooth was divided into thirds from the root furcation to the root apex and the closest third to the root furcation was evaluated. The tissue sections were serial sections and produced approximately 40 sections per specimen, approximately 200 μm from the beginning of the root furcation. The good sections were stained and analysed. Periodontal tissue was then stained with haematoxylin-eosin and tartrate-resistant acid phosphatase (TRAP) using an Acid Phosphatase, Leukocyte Kit (Sigma Diagnostic, St. Louis, MO) and was observed 24 sections for HE and TRAP staining individually ($n = 6$ in each group) with light microscopy. Bone volume at the inter-radicular septum was measured using methods described by Sprogar *et al.* (Figure 1c) (21). The number of osteoclasts in TRAP-stained sections was determined by measuring the distance between the distopalatal root of the maxillary first molar and the surface of the alveolar bone and counting the number of osteoclasts at the alveolar bone surface. The whole area of the cross-section of the distopalatal root of the maxillary first molar were measured. Four fields of per cut were used for TRAP-stained.

Measurement of serum markers of bone turnover

After local RMA injections were administered to WT mice, post-administration blood samples were collected with the mice under anaesthesia by CO_2 just before sacrifice. Serum alkaline phosphatase (ALP) was measured using LIQTEK ALP (Roche Diagnostics K.K., Tokyo, Japan). Serum TRAP concentration was measured using an enzyme-linked immunosorbent assay kit (Immunodiagnostic Systems Ltd., Ontario, Canada).

Statistical analysis

Experimental data are expressed as means and standard errors. The Shapiro–Wilk test was used to confirm data normality and

Tukey's multiple comparison test was used to test for significance. All analyses were performed using Graph Pad Prism v. 7 (Graph Pad Software, Inc., San Diego, CA). $P < 0.05$ was considered significant.

Results

Tooth movement

Figure 2a shows representative micro-CT images acquired after 14 and 21 days of experimental tooth movement. In unloaded teeth, there was no distance between M1 and M2. In the non-RMA groups, movement distance was significantly higher in the 21-day group (0.342 ± 0.033 mm) than in the 14-day group (0.176 ± 0.008 mm) (Figure 2a and 2b). Movement distance was not significantly lower in the 14-day RMA group (0.116 ± 0.004 mm) than in the 14-day non-RMA group (0.176 ± 0.008 mm), but RMA did somewhat inhibit tooth movement. However, movement distance in the 21-day RMA group (0.203 ± 0.006 mm) was significantly lower (40.6 per cent) than that in the 21-day non-RMA group (0.342 ± 0.033 mm) and was similar to that in the 14-day non-RMA group (0.176 ± 0.008 mm) (Figure 2a and 2b).

Findings from haematoxylin-eosin staining and BV/TV measurement in periodontal tissue

In unloaded teeth, BV/TV did not differ significantly between the 14- (54.2 ± 2.5 per cent) and 21-day (50.7 ± 3.1 per cent) non-RMA groups. It also did not differ significantly between the 14- (52.5 ± 2.6 per cent) and 21-day (53.7 ± 4.2 per cent) RMA groups and their respective non-RMA groups (Figure 3a and 3b). In loaded teeth, BV/TV did not differ significantly between the 14- (38.4 ± 2.6 per cent) and 21-day (30.5 ± 1.7 per cent) non-RMA groups, but BV/TV and bone volume at the inter-radicular septum trended lower in the 21-day group. BV/TV did not differ significantly between the 14-day non-RMA (38.4 ± 2.6 per cent) and RMA groups (46.3 ± 3.1 per cent), but trended higher in the RMA group. However, BV/TV was significantly higher (37.4 per cent) in the 21-day RMA group (41.9 ± 0.9 per cent) than in the 21-day non-RMA group (30.5 ± 1.7 per cent), and bone volume was maintained at the inter-radicular septum in a large percentage of the RMA group (Figure 3a and b).

Number of osteoclasts

The number of osteoclasts in alveolar bone around the tooth roots was high in the 14- (3.61 ± 0.2) and 21-day (6.25 ± 0.3) non-RMA groups, and was significantly higher in the 21-day non-RMA group than in the 14-day non-RMA group (Figure 4a and b). Osteoclast number was lower in the 14-day RMA group (2.71 ± 0.5) than in the 14-day non-RMA group (3.61 ± 0.2), but not significantly so. The number of osteoclasts in the 21-day RMA group (3.70 ± 0.2) was significantly lower (40.8 per cent) than that in the 21-day non-RMA group and was similar to that in the 14-day non-RMA group (Figure 4a and b).

Analysis of trabecular bone structure at the tibial neck

Trabecular separation did not differ significantly between the 14- (BV/TV: 19.5 ± 0.8 per cent, Tb.Sp: 129.3 ± 8.3 μm) and 21-day non-RMA groups (22.0 ± 0.9 per cent, 124.8 ± 4.6 μm) (Figure 5a and b). Neither bone volume nor trabecular separation differed significantly between the 14- (20.6 ± 1.1 per cent, 119.8 ± 4.3 μm) and 21-day (21.3 ± 1.1 per cent, 124.3 ± 5.6 μm) RMA groups and their respective non-RMA groups (Figure 5a and b).

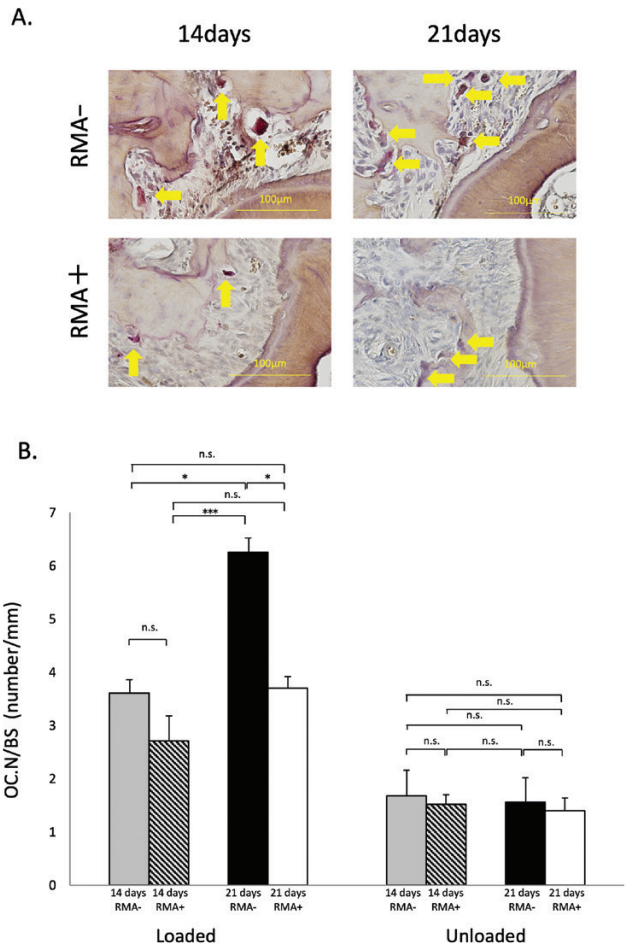

Figure 4. (a)Tartrate-resistant acid phosphatase staining of periodontal tissue after 14 and 21 days of experimental tooth movement (around M1 distopalatal root) (➡: osteoclasts). (b) Osteoclast number (OC.N) as a proportion of bone surface (BS) (number/mm).n.s., not significant; *, $P < 0.05$; ***, $P < 0.001$. RMA+, RMA administration group; RMA−, saline administration group.

Measurement of serum markers of bone turnover

Neither serum TRAP nor serum ALP levels differed significantly between the 14- (TRAP: 2.55 ± 0.15 U/L, ALP: 217.5 ± 17.6 U/L) and 21-day (2.64 ± 0.19 U/L, 247.5 ± 14.6 U/L) non-RMA groups (Figure 6a and b). These also did not differ significantly between the 14- (2.50 ± 0.46 U/L, 217.7 ± 32.6 U/L) and 21-day (2.38 ± 0.13 U/L, 232.4 ± 17.7 U/L) RMA groups and their respective non-RMA groups (Figure 6a and b).

Discussion

Many drugs can be used to control bone metabolism, and local injection of OPG has been reported to have an inhibitory effect on tooth movement (22). In addition, bisphosphonates are one of the most common classes of drug used for the control of bone metabolism. Bisphosphonates are currently used as first-line drugs for osteoporosis. Shoji *et al.* (23) induced experimental tooth movement in OPG$^{-/-}$ mice using the method described by Waldo and Rothblatt (24) and found that treatment with bisphosphonates normalized alveolar bone absorption and tooth movement distance. Bisphosphonates are synthetic analogues of pyrophosphate that are considered to potently inhibit osteoclast bone resorption by acting

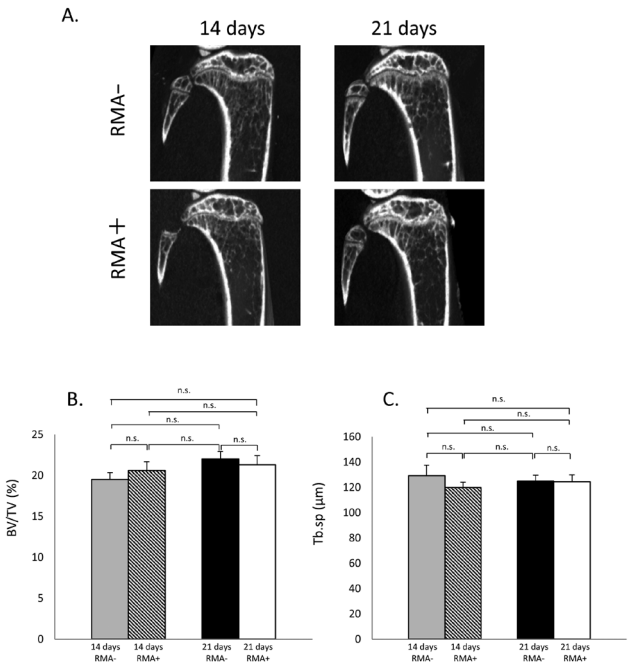

Figure 5. (a) Micro-CT images of tibial neck acquired after 14 and 21 days of experimental tooth movement. (b) Bone volume at the tibial neck (BV/TV). n.s., not significant; RMA+, RMA administration group; RMA–, saline administration group. (c) Trabecular separation at the tibial neck (Tb.Sp). n.s., not significant; RMA+, RMA administration group; RMA–, saline administration group.

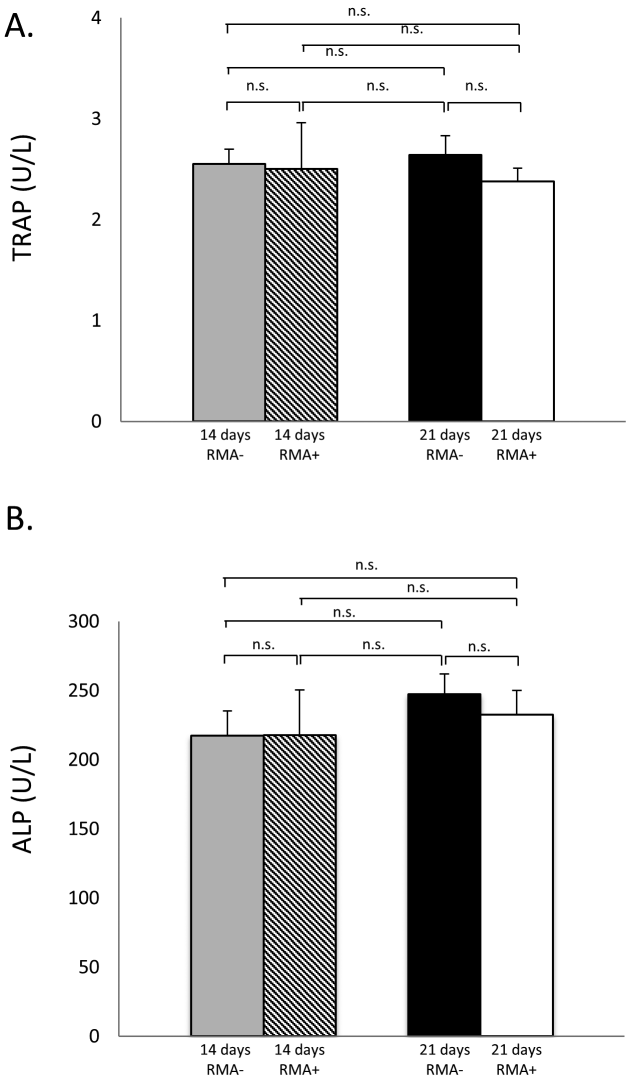

Figure 6. (a) Serum TRAP levels after 14 and 21 days of experimental tooth movement. n.s., not significant; RMA+, RMA administration group; RMA–, saline administration group. (b) Serum ALP levels after 14 and 21 days of experimental tooth movement. n.s., not significant; RMA+, RMA administration group; RMA–, saline administration group.

directly on osteoclasts (25–27) but have been reported to also act on osteoblasts (28). Although the potent effect of bisphosphonates on bone is favourable, their deposition in bone and very long half-life are undesirable, and they have been shown to remain in bone for long periods of up to about 10 years (29, 30). In addition, recent studies have shown that although oral bisphosphonates are effective in reducing the risk of osteoporotic fracture (31, 32), they increase the risk of side effects such as bisphosphonate-related osteonecrosis of the jaw (BRONJ), atypical femoral fracture, and atrial fibrillation (33). Presently, BRONJ is reported in 0.05–0.1 per cent of all patients treated with bisphosphonates, but the incidence is higher in certain groups of patients such as those with malignancies and patients who undergo tooth extraction during treatment (34). Diagnosis and management of BRONJ are also difficult because the aetiology is currently unclear (35–37), hence, our choice of RMA rather than a bisphosphonate to control bone metabolism in our study. Unlike bisphosphonates, whose mechanism of action involves deposition in the bone matrix, RMA, an acidic substance produced by actinomycetes that contains tricarboxylic acid, is selectively taken up by activated osteoclasts in an acidic environment, and selectively induces apoptosis in these cells because activated osteoclasts secrete acid that dissolves bone (13). Therefore, RMA is useful in the treatment of bone metastases because it induces osteoclast apoptosis (14), and it has been shown to be effective in the treatment of osteoporosis in oophorectomized mice and mice fed a calcium-deficient diet (13). Oral RMA is ineffective due to its breakdown by stomach acid and has a very short half-life of just 1 hour (13), and thus, it is believed that even the administration of a high intraoral dose should not cause systemic side effects, which is an advantage.

Upto date, basic research has been conducted to identify the mechanisms of orthodontic tooth movement of different teeth in animals such as dogs, rats, and mice (38). Tanaka *et al.* (15) induced experimental tooth movement in OPG knockout mice for 14 days using an Ni–Ti coil spring and found that repeated intraperitoneal administration of RMA inhibited the absorption of alveolar bone and reduced tooth movement distance in these mice. However, they did not observe inhibition of alveolar bone absorption or tooth movement in WT mice with normal bone metabolism. Chengri *et al.* (19) induced buccal movement of the maxillary incisors for 21 days in mice and found that the number of osteoclasts significantly increased on days 14 and 21 in bone on the buccal aspect of the pressure side, and that movement distance continually increased up to day 21. This indicates that the short tooth movement period of 14 days may have been why Tanaka *et al.* did not observe a significant difference in WT mice. However, very few studies have investigated movement of the first molars in mice for a period longer than 14 days. That is why in this study, we decided to use WT mice to confirm the effects of RMA on tooth movement in mice with normal bone metabolism, and to observe experimental tooth movement induced with

an Ni–Ti coil spring like Tanaka *et al.* (15), but over a longer period of 21 days. This way, we could better evaluate the pharmacological effects of RMA in a model that more closely resembles the treatment of human patients with normal bone metabolism in clinical practice.

In this study, we found that experimental tooth movement was much greater in the 21-day non-RMA group than in the 14-day non-RMA group. Histopathological evaluation showed a significant increase in the number of osteoclasts and a trend towards lower bone volume at the inter-radicular septum. Tanaka *et al.* (15) induced experimental tooth movement in WT mice for 14 days using an Ni–Ti coil spring and found that repeated intraperitoneal administration of RMA reduced the number of osteoclasts, but they found no significant difference in bone volume or tooth movement distance. We observed very similar results to those of Tanaka *et al.* after 14 days of experimental tooth movement (15), but found that local injection of RMA significantly reduced the number of osteoclasts, promoted maintenance of bone volume at the inter-radicular septum in a large percentage of mice, and significantly reduced tooth movement distance after 21 days. This demonstrates that the effects of RMA injection are more readily apparent with long-term tooth movement over 21 days. We also tested for systemic side effects of local injection of RMA in this study. First, to evaluate the effects on distant tissues, we performed blood tests and measured bone volume and trabecular separation at the tibial neck. Blood tests showed no significant difference in serum TRAP or ALP levels between the RMA and non-RMA groups at 14 or 21 days, indicating that local injection of RMA does not produce systemic effects on osteoblasts or osteoclasts. There was also no significant difference in bone volume or trabecular separation at the proximal epiphysis of the tibia between the RMA and non-RMA groups at 14 or 21 days, suggesting that RMA has no effect on distant tissues.

We also evaluated the effects on adjacent tissues by evaluating bone volume and the number of osteoclasts at the inter-radicular septum on the unloaded side. Bone volume and osteoclast count at the inter-radicular septum on the unloaded side did not differ significantly between the RMA and non-RMA groups. Yamashiro *et al.* (39) found that the maximum concentration of ropivacaine detected in the left palatal mucosa of rats administered a local injection of ropivacaine at the right palatal mucosa was only 6 per cent of that at the injection side, indicating the concentration of drugs administered by local intraoral injection mainly localized to the injection side.

Similarly, Kimi *et al.* (40) barely detected ropivacaine in bone at the right incisors after local injection into the buccal mucosa of the right molar in rats, indicating that drugs administered by local intraoral injection also remain localized mesiodistally. However, an experiment on tooth movement in mice showed that local injection of OPG into the mucosa of the maxillary molars inhibits movement of the molars, but may also inhibit movement of the incisors depending on concentration (22). Therefore, more detailed investigation will be necessary to determine the appropriate dose for local intraoral injection of RMA to ensure that inhibition of tooth movement is localized to the target area. This study was a basic research with local injections administered twice daily, which seems to have limited clinical application. In addition, root resorption and periodontal ligament hyalinization were not evaluated and should be examined in future studies. In summary, our results suggest that local injection of RMA can control tooth movement by controlling local osteoclast activity without causing systemic side effects.

Conclusions

Continuous application of orthodontic force to the teeth of WT mice for 14 and 21 days, resulted in tooth movement that was significantly greater after 21 days. Local intraoral injection of RMA inhibited osteoclast activity around the injection site, and this promoted maintenance of alveolar bone at the inter-radicular septum and consequently inhibited tooth movement. In addition, local intraoral injection of RMA did not cause any systemic effects on osteoblasts or osteoclasts, suggesting that it only acts locally on the target tissue. In summary, our results show that local intraoral injection of RMA may be an effective pharmacological approach with no systemic side effects for controlling tooth movement in orthodontic treatment of patients with normal bone metabolism.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Conflicts of interest

All authors declare no potential conflicts of interest with respect to authorship and/or publication of this article.

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