Original Article

Ultrasound enhances the healing of orthodontically induced root resorption in rats

Zhifeng Liu[a]; Juan Xu[b]; Lingling E[b]; Dongsheng Wang[c]

ABSTRACT

Objective: To examine the effect of low-intensity pulsed ultrasound (LIPUS) on orthodontically induced root resorption in rats.

Materials and Methods: Sixty-four male Wistar rats were divided randomly and equally into four groups (n = 16 rats each). The rats were untreated (negative control) or treated with orthodontic tooth movement without (positive control) or with LIPUS at 100 or 150 MW/cm^2 (LIPUS-treated groups). An initial force of 100 g was applied to the areas between the upper right central incisors and the first molars of the rats for 10 days. Eight rats were randomly chosen from each group, and the root resorption index (RRI) was determined with scanning electron microscopy (SEM). Upper first molar-centered mesial-distal tissue slices were generated from the upper first molars and peridentium of the remaining eight rats from each group. Specimen slices were analyzed with hematoxylin-eosin and tartrate-resistant acid phosphatase staining, osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) immunohistochemistry, and optical microscopy. Analyses of cell number, densitometry, and one-way analysis of variance were performed.

Results: The LIPUS-treated groups displayed decreased RRI values, decreased osteoclast numbers and activity levels, and increased OPG/RANKL expression ratios. High-power SEM revealed reparative cementum in the LIPUS-treated samples.

Conclusion: LIPUS regulates osteoclast differentiation via the OPG/RANKL ratio, evoking a reparative effect on orthodontically induced root resorption in rats. (*Angle Orthod.* 2012;82:48–55.)

KEY WORDS: Root resorption; Ultrasound; Osteoclast; RANKL; OPG

INTRODUCTION

Root resorption, a side effect of orthodontic tooth movement,[1] is a widespread concern among orthodontic scholars. The occurrence rate of root resorption ranges between 20% and 100%, with 1.3% of these cases exhibiting severe root resorption greater than 3 mm.[2] Root resorption that occurs as a consequence of orthodontic treatment has all of the features of an inflammatory reaction; therefore, some researchers have proposed that the condition be designated "orthodontically induced inflammatory root resorption." The inflammatory extent of root resorption is determined by many factors such as the invasiveness of the various absorptive cells, tissue sensitivity, and individual variation.[3] Accordingly, it is difficult to predict the development of orthodontically induced root resorption.

The mechanisms of root resorption are thought to be similar to those of bone resorption. In bone resorption processes, mechanical forces induce the activity of the primitive osteoclasts, which differentiate and enter osteoclastogenesis.[4] Cementoclasts, which mediate cement resorption through an acidization/degradation–associated pathway, are similar to osteoclasts in morphology, activity, functions, and features.[5] Tartrate-resistant acid phosphatase (TRAP) staining is used to mark osteoclasts and cementoclasts, and can be used to identify bone and root resorption.

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a type-II transmembrane protein that is

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indispensible for osteoclast development and differentiation. Interactions between RANKL and its transmembrane receptor RANK on the osteoclast precursor surface induce osteoclast differentiation. Osteoprotegerin (OPG, also known as osteoclast inhibitor) is a receptor of tumor necrosis factor and a natural inhibitor of RANKL. Osteoprotegerin inhibits alveolar bone resorption and increases osteoclast apoptosis by inhibiting the ruffling of mature osteoclasts. Osteoclastogenesis depends on the balance of OPG and RANKL expressed by osteoblasts.[6] When the balance is inclined towards OPG, there are fewer active osteoclasts; when inclined towards RANKL, there are more active osteoclasts. According to in vivo and in vitro studies,[7] OPG and RANKL not only adjust terminal osteoclast differentiation, but also influence resorption. Moreover, cementoblasts also can express OPG and RANKL and can modulate osteoclast cytogenesis.[8]

No effective clinical methods are available currently to treat orthodontically induced root resorption. Topical bisphosphonate administration can dose-dependently inhibit root resorption in orthodontically treated rats.[9] However, the clinical utility of bisphosphonate in orthodontics is limited since bisphosphonate is a bone-resorption inhibitor that can affect orthodontic tooth movement.[10]

Transmitted ultrasonography can change the local microenvironmental stress and induce cellular-level biological reactions. Therapeutic ultrasound (intensity 1–3 W/cm^2) produces an obvious thermal reaction in living tissue, eliciting anti-inflammatory effects,[11] stimulating growth factors,[12] and enhancing bone protein expression. Based on their observation that low-intensity pulsed ultrasound (LIPUS) can promote dental tissue formation in rabbits, el-Bialy et al.[13] speculated that LIPUS may be used to treat root resorption. Although this hypothesis has been verified by various cellular studies,[14–16] it has not been tested in animals. Therefore, the purpose of the present study was to explore the effect of LIPUS on root resorption in rats.

MATERIALS AND METHODS

Experimental Animals and Groups

A total of 64 healthy, 8-week-old male Wistar rats (Experimental Animal Center, Academy of Military Medical Sciences), each weighing about 230 g, were divided randomly and equally into four groups (n = 16 rats each). Group I rats were untreated (negative control group). Group II rats were subjected to orthodontic treatment for 10 days only (positive control group). Groups III and IV were subjected to orthodontic treatment combined with LIPUS treatment at 100 MW/cm^2 (Group III) or 150 MW/cm^2 (Group IV) for 10 days (LIPUS-treated experimental groups). All animals were housed individually in plastic cages in a colony room. Animals were fed a standard pellet diet, and water was provided ad libitum.

Animal Model Establishment

The rats were anesthetized with an intraperitoneal injection of 70 mg/kg Dormicum during the setting and adjustment of the orthodontic appliance. A Ni-Ti closed coil spring (Smart Co, China) orthodontic appliance was inserted between the upper incisors and the upper left first molar. The appliance was fixed with a 0.1-mm stainless wire around both teeth.[17] According to the manufacturer's database, the force level of the coil spring after activation is approximately 100 g. The force magnitude was measured with a tension gauge (Changsha Tiantian Dental Co, Ltd) when the appliance was set and at the end of the experiments.

The experimental groups were treated for 20 min/d for 10 consecutive days with LIPUS at a frequency of 1.5 MHz and intensity of 100 or 150 MW/cm.[2] The spring was checked every day to prevent it from dropping off.

Preparation of Specimens for Analyses by Scanning Electron Microscopy or Histology

After 10 days of force application, all 64 rats were killed by neck break. The rats were allocated randomly and equally into two groups for further analysis by scanning electron microscopy (SEM) or histology. Specimens intended for SEM (n = 8 rats from each group) or histologic analysis (n = 8 rats from each group) were fixed in 10% formaldehyde solution for 48 hours.

For SEM analysis, the right upper first molar, including its surrounding bone, was cut as a block. Next, the alveolar bone was removed delicately, so as to avoid any root surface damage. The molars were submerged in 1% sodium hypochlorite to eliminate any periodontal ligament remnants. The molars were dried for 1 week in a 40°C incubator, placed on a retainer, plated with gold, and observed with SEM. Adobe Photoshop ME Version 7 was used to analyze the root resorption index (RRI), defined as the resorbed percentage of the mesial area of the middle palatal root of the first molar (Figure 1).

For histologic analysis, fixed samples were decalcified with 15% EDTA solution for 45 days, dehydrated with alcohol, rendered transparent by xylene, and embedded in paraffin wax. Upper first molar–centered mesial-distal tissue slices were created with a thickness of 3 to 5 μm.

Observation of Staining and Root Resorption

TRAP staining was performed with a reagent kit (Sigma-Aldrich, St. Louis, MO). Areas (400×) around the mesial apex of the middle palatal root were used. The number of osteoclasts that stained positive for TRAP was

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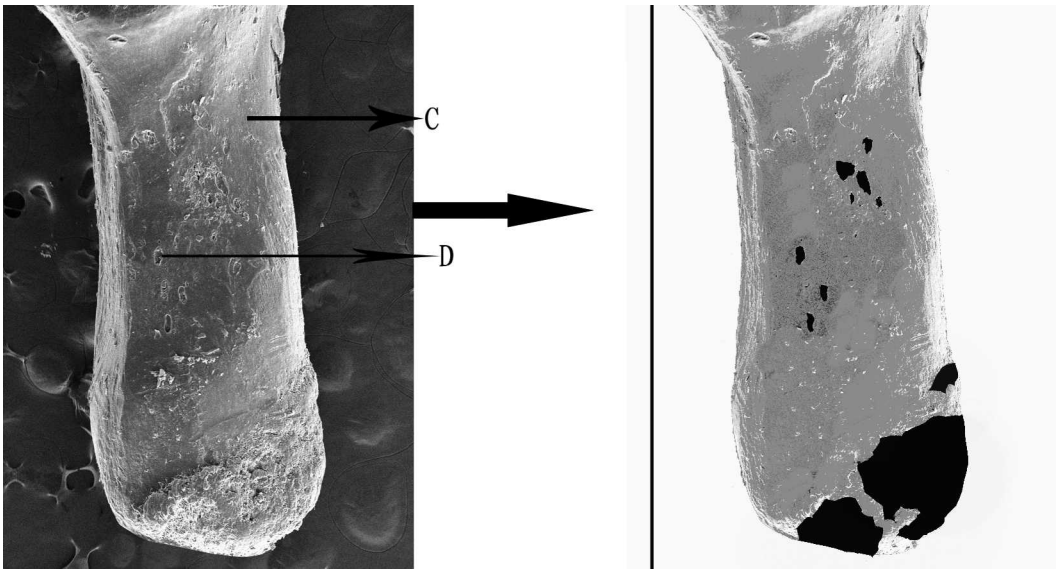

Figure 1. RRI. Black and gray refer to areas of resorption and no resorption, respectively. RRI represents black area / (black area + gray area) * 100%. (D indicates dentin; C, cementum.)

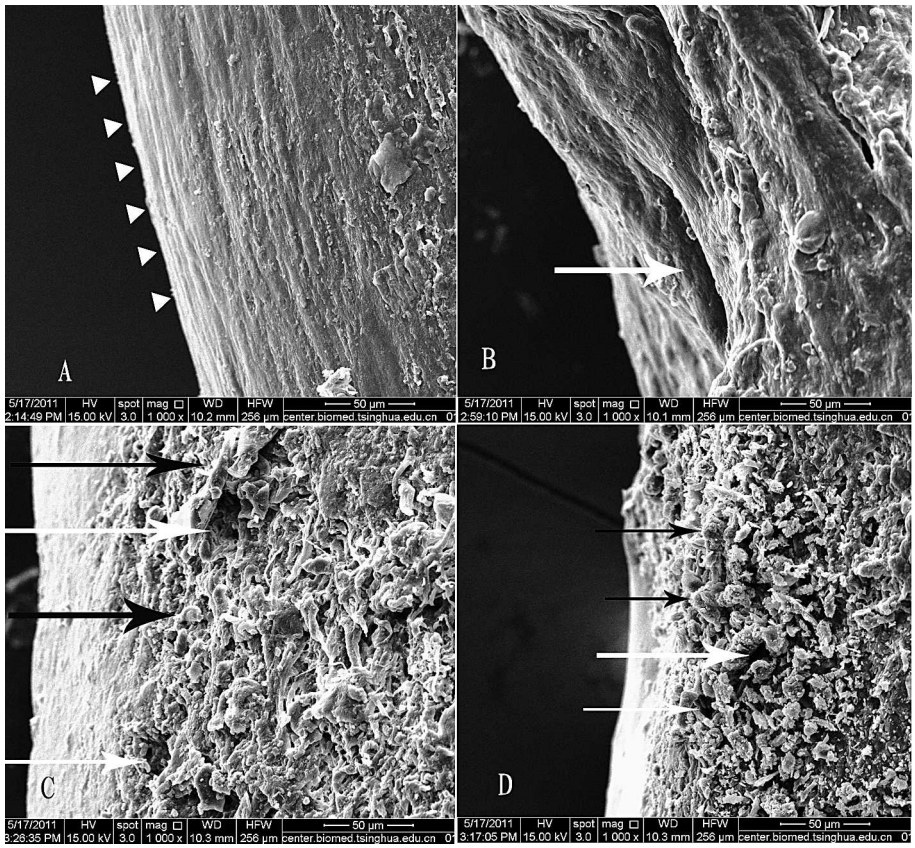

Figure 2. SEM images of control and LIPUS-treated rats. (A) Negative control group showing smooth root surfaces (△) and few root resorption pits. (B) Positive control group showing widespread resorption lacunae with clear-cut yet irregular rims (⇨). (C) 100 MW/cm^2 LIPUS-treated group and (D) 150 MW/cm^2 LIPUS-treated group showing small isolated resorption pits (⟶) and new cementum (⟶) on the root surfaces.

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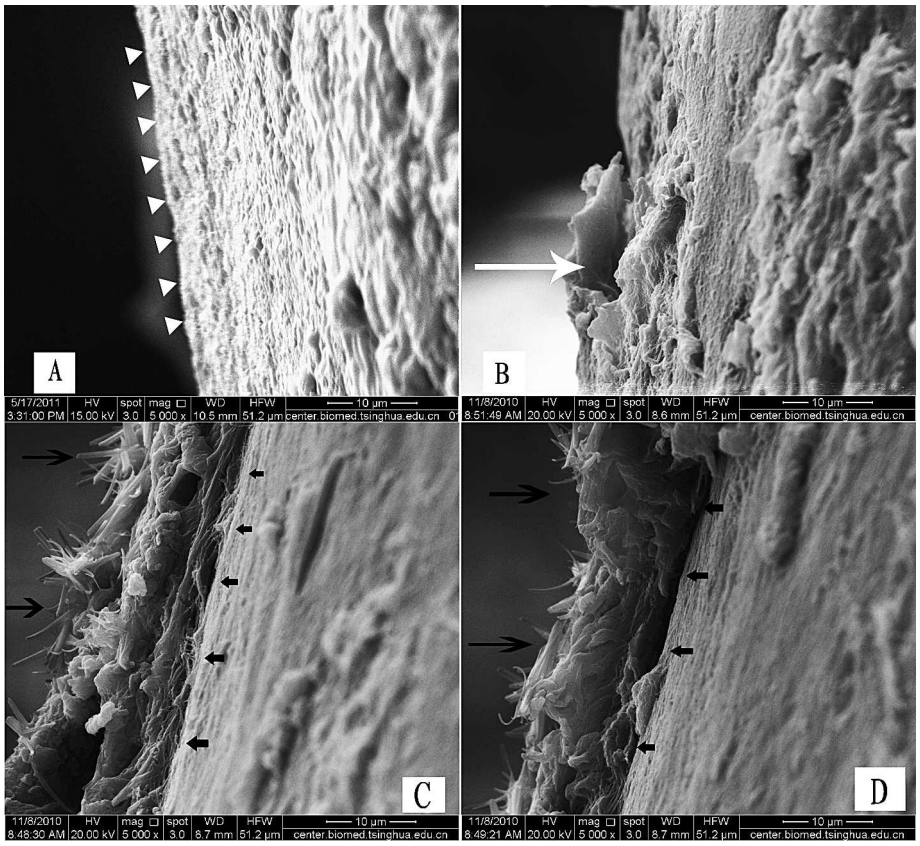

Figure 3. SEM images of control and LIPUS-treated rats. (A) Negative control group showing smooth root surfaces (△) and few root resorption pits. (B) Positive control group showing widespread resorption lacunae with clear-cut yet irregular rims (⇨). (C) 100 MW/cm² LIPUS-treated group and (D) 150 MW/cm² LIPUS-treated group showing increased deposition of new cementum evidently (⟶).

determined as the mean value of three observations by optical microscopy.

Immunohistochemical Staining

Immunological staining was performed with OPG and RANKL polyclonal antibodies (Cruz Company, Santa Cruz), the streptavidin-peroxidase (S-P) immunohistochemical reagent kit, and the diaminobenzidine (DAB) developing box (Beijing Zhongshan Biochemicals Co, Ltd, Zhongshan, China) according to the manufacturers' instructions. In the negative control, phosphate-buffered saline (PBS) was substituted for the primary antibodies. Areas around the mesial apex of the middle palatal root were selected. Image pro-Plus 6.0 was employed to measure the mean optical density (OD) value of the positive products from a single optical microscopy observation (400×).

Table 1. Percentage of Root Resorption for All Groups (Mean ± SD, n = 8)

	Root Resorption, %
Negative control	0.53 ± 0.07
Positive control	22.12 ± 2.82[a]
100 MW/cm² LIPUS-treated	2.80 ± 0.51[a,b]
150 MW/cm² LIPUS-treated	2.78 ± 0.52[a,b]

* LIPUS indicates low-intensity pulsed ultrasound.

[a,b] $P < .05$ by ANOVA compared with negative control (a) or force-only positive control (b).

Statistical Analysis

One-way analysis of variance (ANOVA) was performed with SPSS version 13.0. Multiple intergroup comparisons were performed with the q test. The statistical significance was defined as $P < .05$.

RESULTS

Electron Microscopy

The SEM results revealed smooth root surfaces and few root resorption pits in the negative control group (Figure 2). The positive control group displayed widespread root resorption lacunae. These resorption lacunae had clear-cut yet irregular rims (Figure 2B). Root surfaces in the LIPUS-treated groups were coarser than those in the negative control group, and small isolated resorption pits were seen on the root surfaces (Figure 2C,D). Under high-power lens, a large amount of new cementum was evidently found in both LIPUS-treated groups (Figure 3; Table 1). The total number and

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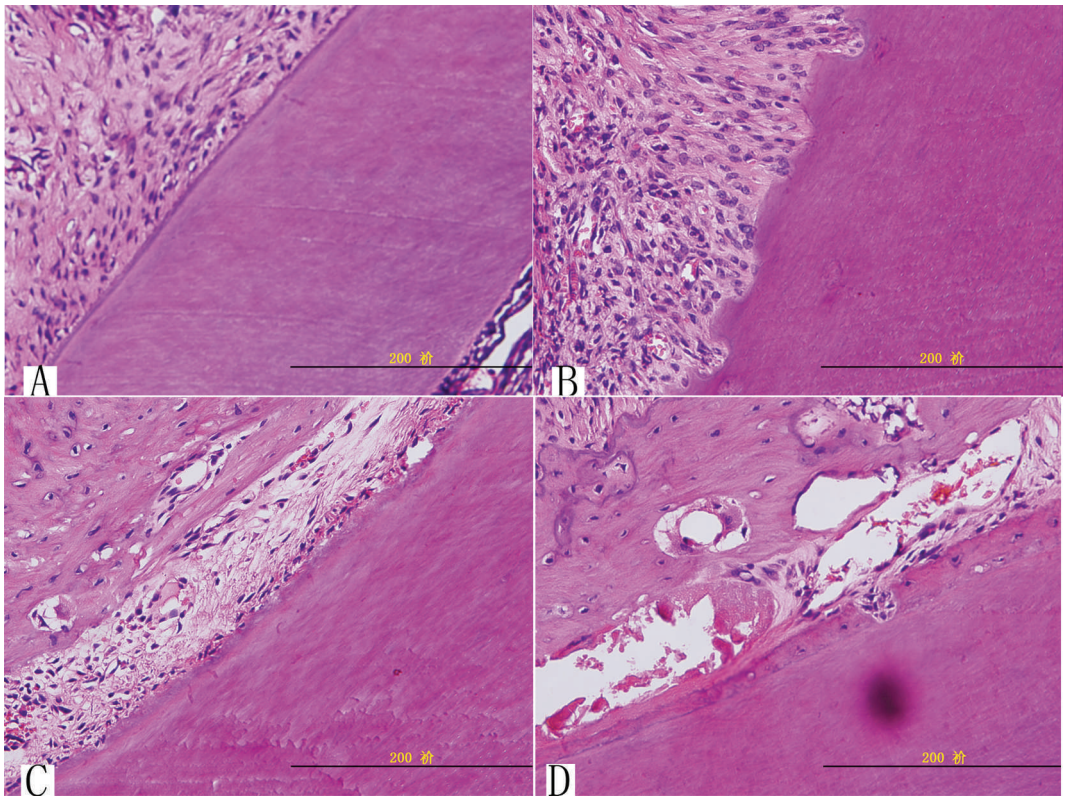

Figure 4. Histologic examination of compressed surfaces (A) Negative control group showing smooth root surfaces. (B) Positive control group showing widespread resorption lacunae. (C) 100 MW/cm^2 LIPUS-treated group and (D) 150 MW/cm^2 LIPUS-treated group showing small isolated resorption pits (HE staining stain, magnification 400×).

area of the resorption lacunae in the LIPUS-treated groups were smaller than those in the positive control group.

Histologic Observations

Staining with hematoxylin-eosin (HE) indicated that root resorption mainly occurred at the root furcation and in the vicinity of the stress-side apex. The LIPUS-treated groups showed less resorption than the positive control group (Figure 4).

TRAP Staining

Except for the negative control group, all groups displayed osteoclasts or osteoclastic cytoplasm at the teeth roots. The alveolar bone area in the three groups stained positive. The multinucleate cells of this area were distributed in the resorption pits of the cementum and the alveolar bone, forming a line along the pit rims. The positive chroma values of TRAP staining in the LIPUS-treated groups were weaker than those in the positive control group (Figure 5; Table 2). These differences between the LIPUS-treated groups and the positive control group were statistically significant. Fewer cells stained positive for TRAP in the LIPUS-treated groups than in the positive control group.

Immunohistochemical Results

The OPG/RANKL expression appeared as a yellow-brown staining in the cytoplasm. The major locations of positive OPG/RANKL expression were the periodontal membrane, cementum, and osteoclasts in the resorption bone pits (Figure 6). Figure 6 displays the optical microscopy images for OPG and RANKL staining. Table 3 summarizes the densitometric analysis of these findings. The mean OD value for OPG of the LIPUS-treated groups was significantly higher than that of the positive control group. The OPG OD values of the two LIPUS-treated groups also were significantly different from each other. The mean RANKL OD value of the LIPUS-treated groups was significantly lower than that of the positive control group. The RANKL OD values of the two LIPUS-treated groups were significantly different from each other.

DISCUSSION

In this study, we explored the effect of LIPUS on root resorption in rats. The RRI values were reduced in the LIPUS-treated groups. High-power SEM revealed newly developed cementum in the LIPUS-treated groups. Fewer osteoclasts were seen in the

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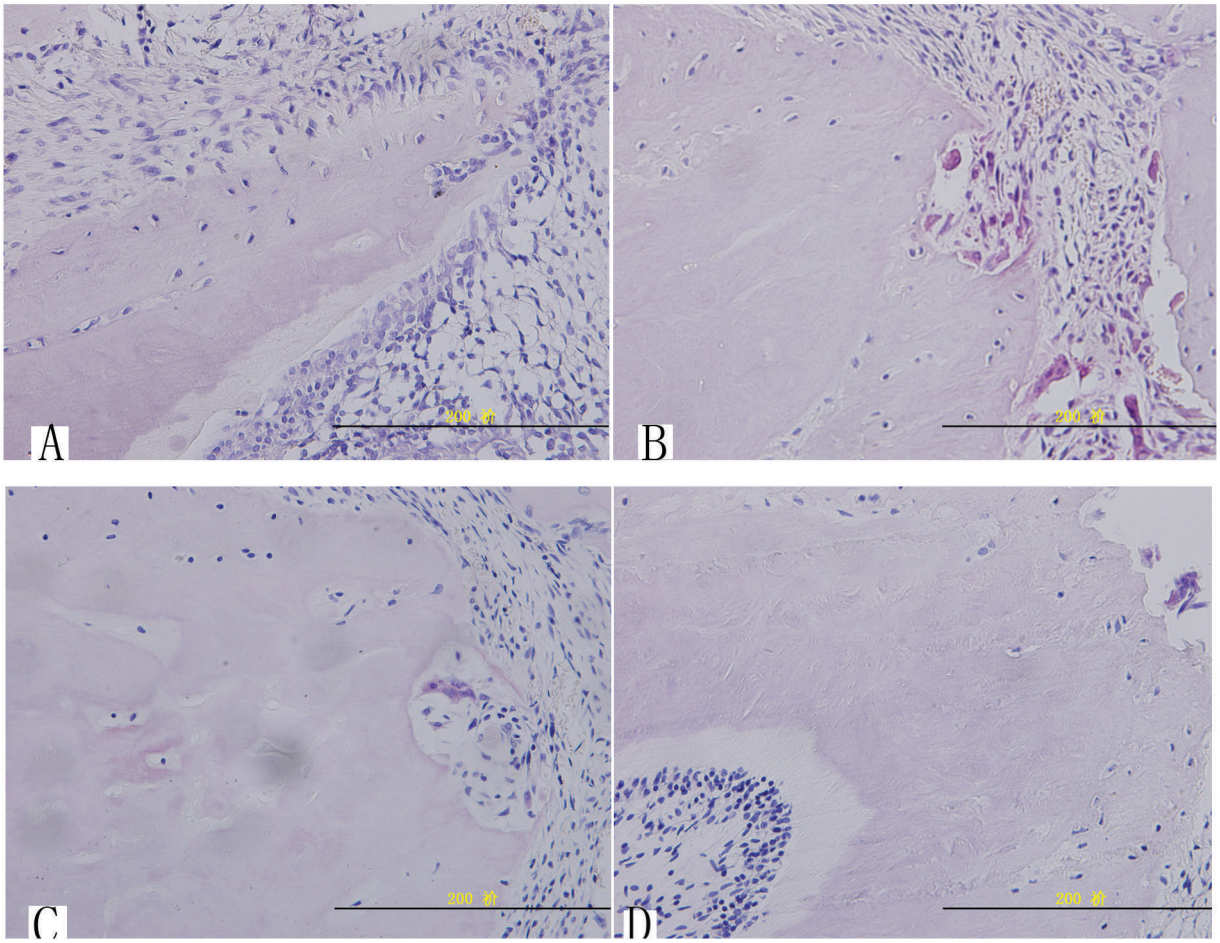

Figure 5. TRAP staining of the rat molar tooth root. (A) Negative control group showing few osteoclasts in the apex. (B) Positive control group showing substantial numbers of osteoclasts in the apex. (C) 100 MW/cm^2 LIPUS-treated group and (D) 150 MW/cm^2 LIPUS-treated group showing fewer osteoclasts in the apex than in the positive control group (magnification 400×).

groups treated with LIPUS than in the positive control group, and overall the cells that were seen were less active. These results indicate that root resorption was less active in the LIPUS-treated groups. Finally, 100 MW/cm^2 LIPUS was more effective than 150 MW/cm^2 LIPUS, consistent with previous reports that the stimulatory effect of LIPUS is dose-dependent.[18]

Treatment with LIPUS increased OPG and decreased RANKL expression in the experimental groups, thereby reducing the number and activity of osteoclasts and naturally reducing root resorption. By treating cementoblasts with high-intensity (150 MW/cm^2) or low-intensity (30 MW/cm^2) ultrasonic radiation, Dalla-Bona[16] previously found that LIPUS increases OPG expression but does not affect RANKL expression. There are at least two potential reasons for this observed difference in results. First, LIPUS can influence stem cells in the periodontal membrane and can induce interactions among cells. For example, exposure of human umbilical cord–derived mesenchymal stem cells to LIPUS increases the mesenchymal stem cell yield by promoting release and enhancing proliferation.[19] Second, the mechanical stimulus elicits the strong release of inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-β (IL-β), and prostaglandin E2 (PGE2), from the weakened periodontal ligament. These inflammatory factors can stimulate RANKL expression. LIPUS can reduce the levels of these inflammatory factors, TNF-α and IL-β, thereby reducing RANKL expression and osteoclast differentiation.

Table 2. Osteoclast Numbers in All Groups (Mean ± SD, n = 8)

	Number of Osteoclasts
Negative control	0.6 ± 0.7
Positive control	6.6 ± 1.1[a]
100 MW/cm^2 LIPUS-treated	4.0 ± 0.9[a,b]
150 MW/cm^2 LIPUS-treated	4.1 ± 1.2[a,b]

* LIPUS indicates low-intensity pulsed ultrasound.

[a,b] $P < .05$ by ANOVA compared with negative control (a) or force-only positive control (b).

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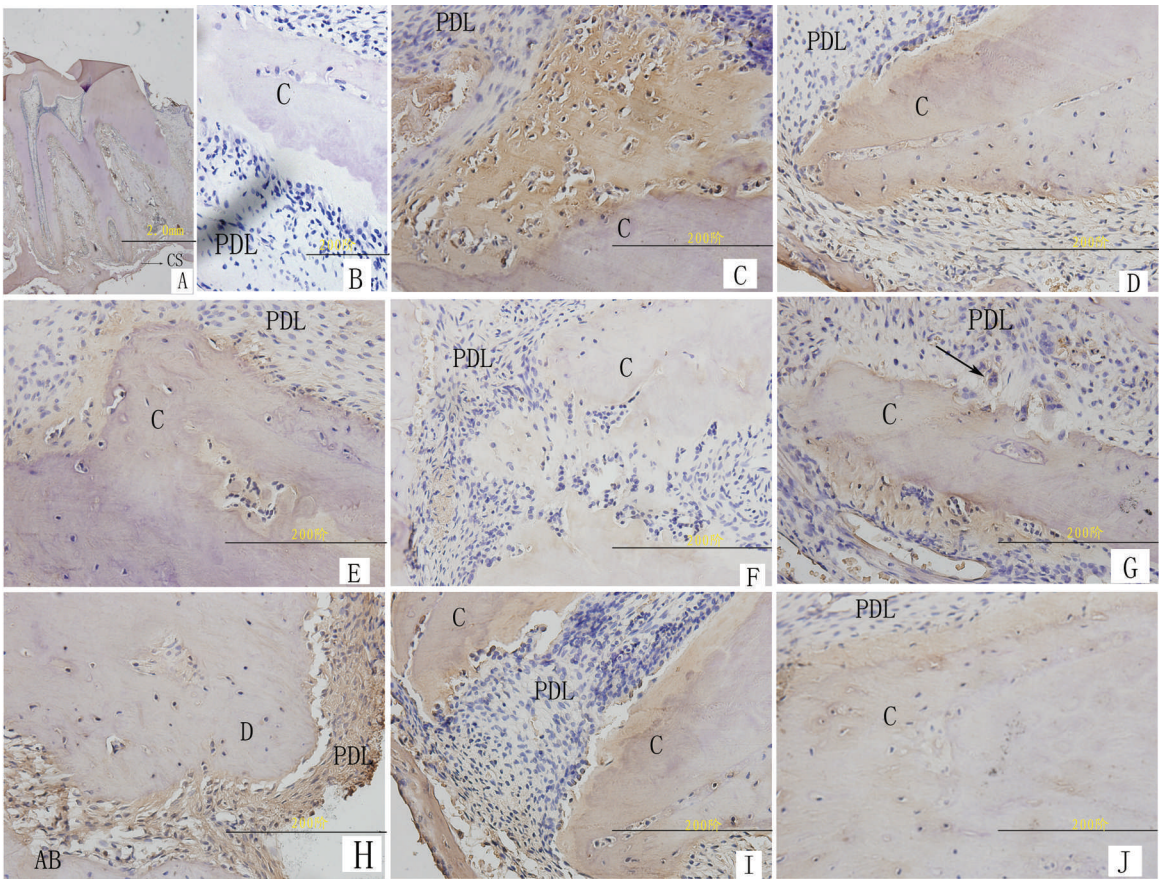

Figure 6. RANKL immunohistochemical staining of the rat molar tooth root. (A) Apical region of the middle palatal root of orthodontic tooth movement. (B) Negative control experiment using nonimmune IgG instead of the primary antibody. (C-F) Immunoreactivity for RANKL at day 10. Specimens are shown after treatment with mechanical force (100 g) without (C) or with LIPUS at an intensity of 100 MW/cm^2 (D) or 150 MW/cm^2 (E). Untreated specimens (F) show weak immunoreactivity for RANKL compared with other groups, which show strong staining for RANKL on the compressed side. (G-J) Immunoreactivity for OPG on day 10. Specimens are shown after treatment with mechanical force (100 g) without (G); arrows refer to osteoclasts in resorption lacunae) or with LIPUS at an intensity of 100 MW/cm^2 (H) or 150 MW/cm^2 (I). Untreated specimens (J) show weak immunoreactivity for OPG compared with the other groups. PDL indicates periodontal ligament; C, cementum. Magnification is 40× (A) or 400× (B-J).

Prolonged treatment with LIPUS previously was shown to promote bone and incisor growth and repair as well as the rate of tooth eruption during mandibular distraction.[20] Exposure of cementoblasts to LIPUS affects the mRNA expression of alkaline phosphatase and increases cellular calcium levels, which regulate the mineralization process. However, LIPUS had no effect on cell proliferation in this previous study.[14] Recently, LIPUS was shown to stimulate MSC (mesenchymal stem cells) differentiation along cartilage and osteogenic lineages.[21] These findings suggest that therapeutic ultrasound may be advantageous for stem cell and tissue engineering applications, and has great potential in hard tissue repair and regeneration.

The different phylogenic status between humans and lower animals such as mice, rats, and rabbits makes it difficult to extrapolate results from animal models to the clinic. The growth pattern of cementum

Table 3. Mean OPG and RANKL OD Values for All Groups (Mean ± SD, n = 8)

	OPG OD Values	RANKL OD Values
Negative control	0.0325 ± 0.0017	0.0304 ± 0.0018
Positive control	0.0437 ± 0.0021[a]	0.0898 ± 0.0029[a]
100 MW/cm^2 LIPUS-treated	0.1007 ± 0.0022[a,b]	0.0564 ± 0.0024[a,b]
150 MW/cm^2 LIPUS-treated	0.0675 ± 0.0032[a,b,c]	0.0642 ± 0.0028[a,b,c]

* LIPUS indicates low-intensity pulsed ultrasound; OPG, osteoprotegerin; OD, optical density; RANKL, receptor activator of nuclear factor kappa-B ligand.

[a,b,c] $P < .05$ by ANOVA compared with negative control (a), force-only positive control (b), or 100 MW/cm^2 LIPUS-treated group (c).

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in lower animals involves continuous eruption, with cementum being formed throughout their lifetime. However, the study of root resorption in an adequate sample size of higher animals (eg, monkeys) is prohibitively expensive. As a result, the rat model for root resorption remains widely used.

Therapeutic ultrasound can be used to stimulate the expression of bone proteins (osteonectin, osteopontin, and bone sialoprotein) in a dose-dependent manner.[22] The frequency and intensity of ultrasound used for imaging the human brain (7.5–20 MHz) are much higher than those used for LIPUS (1.5 MHz); therefore, the latter is much safer. Ultrasound also is being used to diagnose early stages of cancer. Thus, ultrasound is considered to be noncarcinogenic and has no known deleterious effects.[23] These findings may help promote the development of convenient, noninvasive devices to prevent tooth resorption and improve human health and quality of life. Additional studies on this process are recommended.

CONCLUSION

- LIPUS has a reparative effect on orthodontic root resorption in rats by modulating the OPG/RANKL ratio and osteoclast differentiation. Therefore, LIPUS may serve as a potential treatment for orthodontically induced root resorption.

REFERENCES

1. Goss SA, Johnston RL, Dunn F. Comprehensive compilation of empirical ultrasonic properties of mammalian tissues. *J Acoust Soc Am*. 1978;64:423–457.
2. Owman-Moll P, Kurol J. Root resorption after orthodontic treatment in high-and low-risk patients: analysis of allergy as a possible predisposing factor. *Eur J Orthod*. 2000;22: 657–663.
3. Brezniak N, Wasserxtein A. Orthodontically induced inflammatory root resorption. Part I: the basic science aspects. *Angle Orthod*. 2002;72:175–179.
4. Blair HC, Athanasou NA. Recent advances in osteoclast biology and pathological bone resorption. *Histol Histopathol*. 2004;19:189–199.
5. Matsumoto Y. Morphological and functional properties of odontoclasts on dentine resorption. *Kokubyo Gakkai Zasshi*. 1994;61:123–143.
6. Ishii M, Iwai K, Koike M, et al. RANKL-induced expression of tetraspanin CD9 in lipid raft membrane microdomain is essential for cell fusion during osteoclastogenesis. *J Bone Miner Res*. 2006;21:965–976.
7. Sasaki T. Differentiation and functions of osteoclasts and odontoclasts in mineralized tissue resorption. *Microsc Res Tech*. 2003;61:483–495.
8. Boabaid F, Berry JE, Koh AJ, Somerman MJ, McCauley LK. The role of parathyroid hormone-related protein in the regulation of osteoclastogenesis by cementoblasts. *J Periodontol*. 2004;75:1247–1254.
9. Igarashi K, Mitani H, Adachi H, Shinoda H. Inhibitory effect of topical administration of a bisphosphonate (risedronate) on root resorption incident to orthodontic tooth movement in rats. *J Dent Res*. 1994;75:1644–1649.
10. Alatli I, Hellsing E, Hammarstrom L. Orthodontically induced root resorption in rat molars after 1-hydroxyethylidene-1, 1-bisphosphonate injection. *Acta Odontol Scand*. 1996;54: 102–108.
11. Iashchenko LV, Ostapiak ZN, Semenov VL. The humoral mechanisms of the action of ultrasound in inflammatory lung diseases (an experimental study). *Vopr Kurortol Fizioter Lech Fiz Kult*. 1994;2:20–22.
12. Young SR, Dyson M. The effect of therapeutic ultrasound on angiogenesis. *Ultrasound Med Biol*. 1990;16:261–269.
13. el-Bialy TH, el-Moneim Zaki A, Evans CA. Effect of ultrasound on rabbit mandibular incisor formation and eruption after mandibular osteodistraction. *Am J Orthod Dentofacial Orthop*. 2003;124:427–434.
14. Dalla-Bona DA. *Effects of Ultrasound on the Metabolism of Cementoblast in Vitro* [PhD thesis]. Hiroshima, Japan: Hiroshima University; 2007.
15. Inubushi T. Effects of ultrasound on the proliferation and differentiation of cementoblast lineage cells. *J Periodontol*. 2008;79:984–990.
16. Dalla-Bona DA. Cementoblast response to low- and high-intensity ultrasound. *Arch Oral Biol*. 2008;53:318–323.
17. Yoshimatsu M, Shibata Y, Kitaura H, Chang X, Moriishi T, Hashimoto F, Yoshida N, Yamaguchi A. Experimental model of tooth movement by orthodontic force in mice and its application to tumor necrosis factor receptor-deficient mice. *J Bone Miner Metab*. 2006;24:20–27.
18. Shimazaki A, Inui K, Azuma Y, Nishimura N, Yamano Y. Low-intensity pulsed ultrasound accelerates bone maturation in distraction osteogenesis in rabbits. *J Bone Joint Surg Br*. 2000;82-B:1077–1082.
19. Yoon JH. Introducing pulsed low-intensity ultrasound to culturing human umbilical cord-derived mesenchymal stem cells. *Biotechnol Lett*. 2009;31:329–335.
20. el-Bialy TH, Royston TJ, Magin RL, Evans CA, Zaki Ael M, Frizzell LA. The effect of pulsed ultrasound on mandibular distraction. *Ann Biomed Eng*. 2002;30:1251–1261.
21. Cui JH, Park K, Park SR, Min BH. Effects of low-intensity ultrasound on chondrogenic differentiation of mesenchymal stem cells embedded in polyglycolic acid: an in vivo study. *Tissue Eng*. 2006;12:75–82.
22. Harle J, Salih V, Mayia F, Knowles JC, Olsen I. Effects of ultrasound on the growth and function of bone and periodontal ligament cells in vitro. *Ultrasound Med Biol*. 2001;27:579–586.
23. Turnbull DH, Foster FS. In vivo ultrasound biomicroscopy in developmental biology. *Trends Biotechnol*. 2002;20: S29–S33.

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