
学位論文

Effects of Bisphosphonates on Root Resorption during Experimental Tooth Movement

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Key words : bisphosphonate, root resorption, tooth movement

Abstract : The aim of the present study was to investigate the effect of the local administration of three kinds of bisphosphonates -etidronate, alendronate, and pamidronate- on orthodontic root resorption. Both the right and left molars of 7-week-old male Sprague Dawley rats were experimentally moved by interproximal insertion of the elastic band according to Waldo's method. Each bisphosphonate solution was injected into the palatal subperiosteum area adjacent to the upper first molar every third day during experimental period. Rats were sacrificed after 1, 3, 7, and 14 days of elastic band insertion, and samples prepared from tissues were histomorphometrically analyzed by light or scanning electron microscopy (SEM). The SEM analysis revealed that the local injection caused a significant reduction in mechanically induced root pit formation in all bisphosphonate treatment groups after 14 days. The root pit formation was parallel with the reduction in the number of odontoclasts in histological examination for tartrate-resistant acid phosphatase activity. However, on day 3, treatment with etidronate and alendronate transiently increased the root pit formation due to the increase of the odontoclast number. Immunohistochemical study was performed to examine the effect of these bisphosphonates on the expression of proinflammatory cytokines, i.e., interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6). On day 3, in etidronate- and alendronate-treatment groups, the strong immunoreactive signals of IL-1 β and TNF- α were observed in odontoclasts, osteoclasts, and periodontal ligament cells compared with control groups. Pamidronate treatment did not affect the expression of IL-1 β , TNF- α , and IL-6 during whole experimental period. Thus, topical application of bisphosphonates following experimental induction of tooth movement in rats reduced the root resorption and the number of odontoclasts. These results suggest that bisphosphonates may be useful for prevention of root resorption of teeth during orthodontic tooth movement. Moreover, the effect of etidronate and alendronate via IL-1 β and TNF- α expression might be associated with odontoclast and osteoclast formation.

Introduction

Dental root resorption is a common idiopathic problem associated with orthodontic treatment and has recently received considerable attention because of medicolegal exposure. Odontoclasts, which are responsible for the resorption of teeth, have been thought to be derived from mononuclear precursors in the monocyte-macrophage lineage, and demonstrate the similar characteristics to osteoclasts responsible for bone resorption^{1, 2)}. There have been several studies on the etiology and the mechanism of root resorption in both biological and clinical aspects^{1, 3-5)}. At present, however, root resorption associated with orthodontic treatment seems to be unpredictable and often unavoidable. If undesirable root resorption could be prevented, orthodontic treatment would be less complex and more secure.

Bisphosphonates selectively inhibit osteoclast function, and have been used to treat various metabolic bone diseases associated with excessive bone resorption⁶⁻⁸⁾. Bisphosphonates have a P-C-P bond instead of the P-O-P bond of inorganic pyrophosphate, which makes them resistant to enzymatic degradation, and give them high affinity for hydroxylapatite⁹⁻¹⁵⁾. Studies on the relationships between the structure and activity in bisphosphonates indicate that their potency and mode of action vary depending on the side-chain on the carbon atom of the P-C-P bond, a common structure of bisphosphonates. Recent reports have revealed that bisphosphonates containing a nitrogen atom in the side-chain (N-containing bisphosphonate) are more potent than those without a nitrogen atom (non-N-containing bisphosphonates), and that they can inhibit the production of isoprenoid compounds (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) in the mevalonate pathway and, hence, prevent protein prenylation in osteoclasts⁶⁻¹²⁾. It has also been shown that non-N-containing bisphosphonates can incorporate into ATP analogues and act by inhibiting protein synthesis and inducing apoptosis in osteoclasts^{13, 14)}.

The present study was undertaken to examine the effect of topical administration of bisphosphonates, etidronate as non-N-containing bisphosphonate (Fig. 1A), as well as alendronate and pamidronate as N-containing bisphosphonates (Fig. 1B, C), on root resorption during experimental tooth movement in rats as the first step to determine future clinical application of these compounds.

Materials and methods

1. Materials

The bisphosphonates -etidronate, alendronate, and pamidronate- were obtained from Dainippon Sumitomo Pharma Co (Osaka, Japan), Banyu Pharmaceutical Co (Tokyo, Japan), and Novartis Pharma K.K. (Tokyo, Japan),

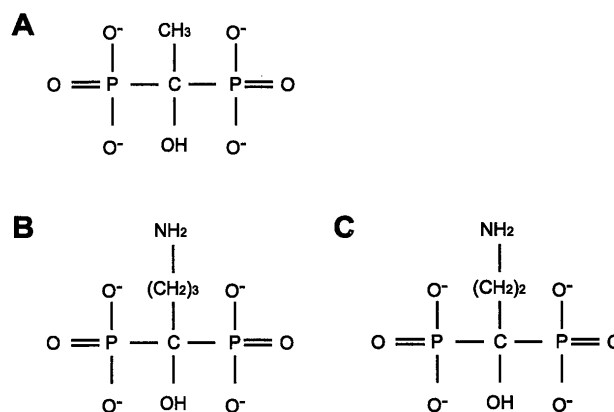


Fig. 1 Chemical structure of the bisphosphonates investigated in this study. (A) etidronate. (B) alendronate. (C) pamidronate.

respectively. Seven-week-old male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan), weighting 240-260 g, were kept in a room at 25°C with a 12-hour/12-hour light-dark cycle. The animals were treated in compliance with the regulation for the use of experimental animals of the Animal Care and Use Committee of the University of Tokushima.

2. Experimental model for tooth movement

Rats were anesthetized with sodium pentobarbital (15 mg/kg intraperitoneally). According to the method of Waldo and Rothblatt¹⁵⁾, a piece of orthodontic elastic bands (0.6 mm thick; GAC International Inc., Central Islip, NY, USA) was inserted interproximally between the upper first and second molars on both sides (Fig. 2).

3. Bisphosphonate treatment

Bisphosphonates were dissolved in saline solution (0.9% NaCl). A solution of etidronate, alendronate, or pamidronate was injected into the palatal subperiosteum area adjacent to the right and left upper molars in elastic-inserted rats at a dose of 1.0 or 2.5 mg/kg body weight according to the methods described previously¹⁶⁻²⁰⁾. The injection began one day before the elastic band insertion and was performed every third day during experiment period. The rats with injection of saline vehicle served as the controls.

4. Tissue preparation

After 1, 3, 7, and 14 days of elastic band insertion, the rats were anesthetized and fixed by cardiac perfusion with freshly made 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. For scanning electron microscope (SEM) analysis, the upper first molars were extracted, and soft tissues were dissected by maceration in 2.5% NaOCl solution for 5 min.

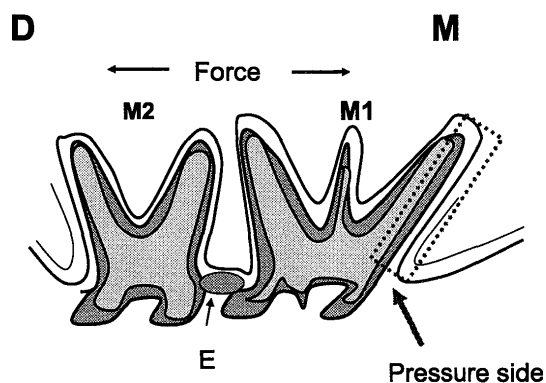


Fig. 2 Diagram of the method of experimental tooth movement. A piece of elastic band is inserted between the upper first molar (M1) and the second molar (M2). The rectangle indicates the measurement area. E, elastic; M, mesial side; D, distal side.

The teeth were rinsed in distilled water, briefly dehydrated in graded ethanol baths, then dried overnight in air. The specimens were mounted on aluminum stubs and coated with gold (150 Å thickness) using the IB-3 ion coater (Eiko Engineering Co, Ibaraki, Japan) for examination by SEM (JSM5300, JOEL, Tokyo, Japan). For light microscopy, the maxillary bones with the surrounding tissues were dissected and fixed in the same fixative overnight at 4°C. They were then demineralized in 19% EDTA (pH 7.4) for 3-4 weeks until the specimen became soft for further processing. The demineralized tissues were dehydrated, and embedded in paraffin. The tissue blocks were cut into 3 µm-thick mesiodistal serial sections and the sections were mounted on 3-(triethoxysilyl)-propylamine-coated slides.

5. Determination of root resorption

To examine the root resorption area, sequential electron micrographs were taken from each specimen along the long axis of the root of the standardized manner (Fig. 3A). Three sequential electron micrographs at 100 x magnification were determined at right angle to the root surface, starting from the cemento enamel junction and covering area 425 x 840 µm of each root. The area occupied by lacuna on the electron micrographs was measured using the Planimeter (Tamaya Technics Inc., Tokyo, Japan). The areas of investigation were the mesial surfaces on the mesial roots of the first molars, serving as the pressure side.

6. Cell measurements

The observed areas of the periodontal tissues were the mesial surfaces on the mesial roots of the first molars, serving

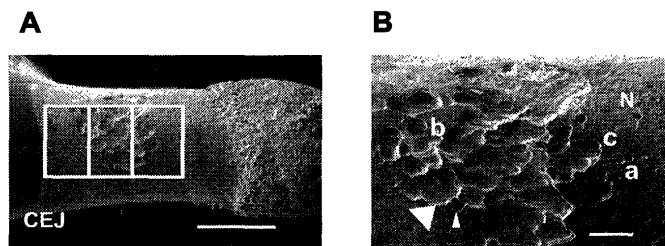


Fig. 3 (A) A low power scanning electron micrograph illustrating the axial sequence of micrographs recorded from each specimen through the center of the defect. Day 3 in control group. Bar=500 µm.

(B) A high power scanning electron micrograph showing distinct types of lacuna on the root surface: small isolated (a), shallow (b), and deep (c). Small lacuna (small arrow) in continuity with superficial resorption bay (large arrow). Day 3 in control group. Bar=100 µm. N, normal cementum; CEJ, cemento enamel junction.

as the pressure side (Fig. 2). The rectangular area of 250 x 1000 µm was marked out on the mesial root of the first molar (Fig. 2). The number of odontoclasts and osteoclasts were counted within the designated area. To identify odontoclasts and osteoclasts, the sections were stained for tartrate-resistant acid phosphatase (TRAP) activity using the Acid phosphatase leukocyte kit (Sigma Chemical Co, St. Louis, MO, USA) according to the manufacturer's instructions, then counterstained with hematoxylin. Odontoclasts were defined as multinucleated TRAP-positive cells on the root surface or in the root resorptive lacunae.

7. Immunohistochemistry

Anti-rat interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) antibodies were purchased from R & D Systems Inc (Minneapolis, MN, USA). The sections were deparaffinized, and then soaked in methanol containing 3% hydrogen peroxide. Following a wash in 10 mM phosphate-buffered saline (PBS) (pH 7.2), the sections were incubated with 1% bovine serum albumin (BSA) for 30 min at room temperature to block non-specific binding of the antibody. Each primary antibody was diluted at 1:500 in PBS containing 0.1% BSA. The sections were probed with the primary antibody for 16 h at 4°C. The primary antibody was detected using the Histofine SAB kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocols. Following incubation streptavidin-horseradish peroxidase, the sections were developed using the Histofine simple stain DAB kit (Nichirei). The coloring reaction was stopped by rinsing the slides with distilled water, and then the sections were counterstained with 3% methyl green. As the negative

controls, nonimmune serum was used instead of the primary antibody for the reaction.

8. Statistical analysis

For all parameters considered, a mean value of the histomorphometrically evaluated sections was obtained per animal. The paired t-test was used to evaluate the significance of differences in root resorption and cell count between the bisphosphonate-treated groups and the control groups. Statistical evaluations were carried out with statistical analysis software package (SPSS version 10.0J, SPSS Japan, Tokyo, Japan). Data are reported as the mean \pm standard error of the mean.

Results

1. SEM observation

Dental root resorption was observed along the mesial surface on the mesial root of the first molar due to experimental tooth movement (Fig. 3A). SEM examination showed morphologically distinct types of lacunae (Fig. 3B): small isolated lacuna, wide shallow resorption bays with no detectable dentinal tubules, and deep resorption lacunae extending into dentine. Three different types of resorption defects are thought to be due to the different resorptive potential in the root-resorbing cells. In the present study, the area occupied by each resorption type did not significantly vary in the control and bisphosphonate-treated groups during the experimental period (data not shown). As shown in Fig. 4, the resorption area of root surface was increased by mechanical stress in a time-dependent manner. To investigate the inhibitory effect of different types of bisphosphonates, the area of pits on the root surfaces were examined in rats topically injected with etidronate, alendronate, and pamidronate. The root resorption area induced by mechanical forces was significantly inhibited after 14 days at doses of 1 and 2.5 mg/kg bisphosphonates, and the similar patterns in the inhibitory effect of these bisphosphonates on root pit formation was shown (Fig. 4). On the other hand, it was interestingly observed that the area of root resorption was significantly increased in etidronate and alendronate treatment groups (2.5 mg/kg) compared with the control groups on day 3 (Fig. 4A, B).

2. Changes in the number of TRAP-positive cells

Histological examination for TRAP staining was performed to identify odontoclasts and osteoclasts. Odontoclasts on the root surfaces and osteoclasts on the alveolar bones were positively stained for TRAP activity, and TRAP-positive mononuclear cells that were considered as precursors of odontoclasts and osteoclasts were observed

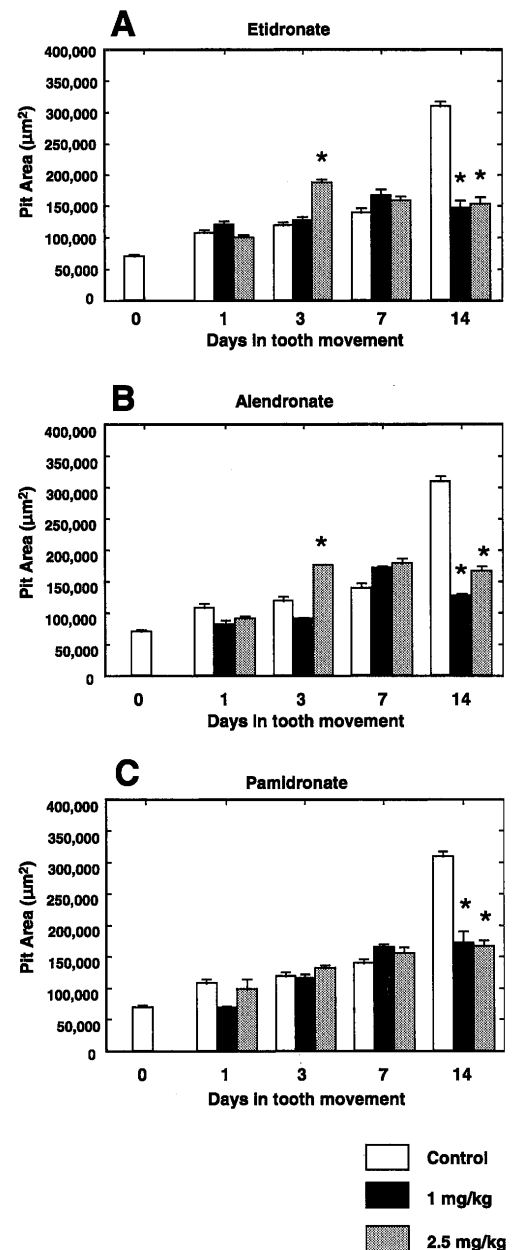


Fig. 4 Variation in resorption area on the root surface in rats treated with etidronate (A), alendronate (B), and pamidronate (C) during experimental tooth movement. Each column and bar represents the mean \pm standard error of the mean ($n=5$ or 6); * $p<0.05$ vs control.

in periodontal ligament (PDL) (Fig. 5). Overall, the morphometric results corroborated the qualitative histological observations, especially regarding to the number of TRAP-positive cells. As shown in Fig. 6, the number of odontoclasts as well as osteoclasts was gradually increased in response to mechanical forces in control group. On day 14, all bisphosphonate-treatment significantly inhibited the formation of odontoclasts, osteoclasts, and their precursors. Thus, the changes in the number of odontoclasts on root surfaces

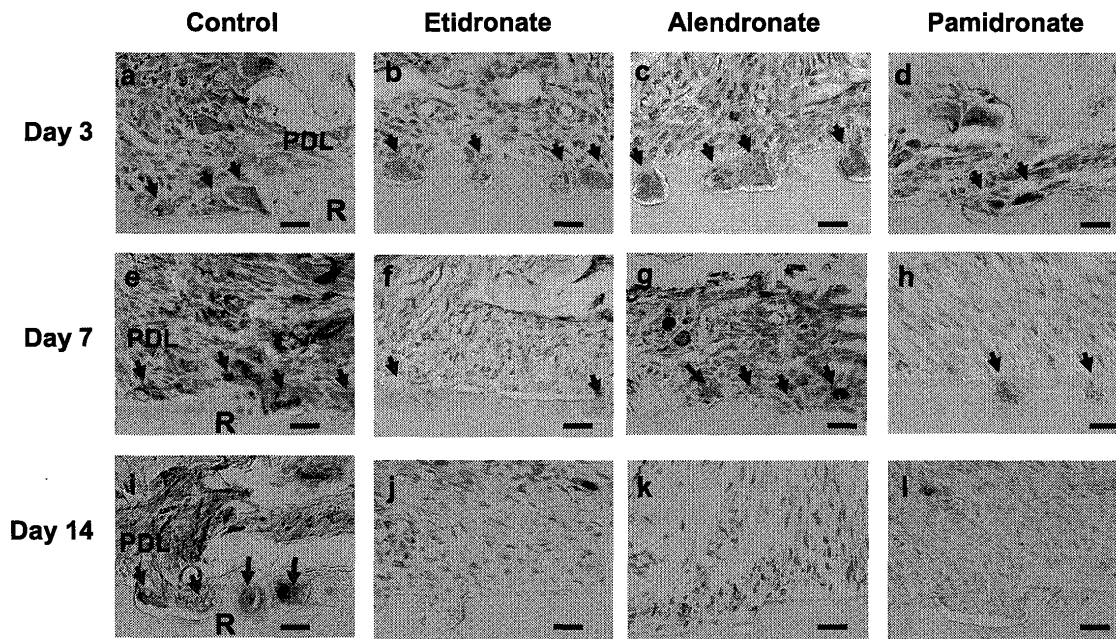


Fig. 5 TRAP staining in sections of a rat dentoalveolar unit treated with 2.5 mg/kg of bisphosphonates - etidronate (b, f, j), alendronate (c, g, k), and pamidronate (d, h, i) - during experimental tooth movement. Control groups (a, e, i) received the same dose of saline vehicle. Tissues prepared at 3 (a - d), 7 (e - h), and 14 (i - l) days after insertion of the elastic band. The arrow indicates odontoclasts on the root surface. Magnification 400 X. Bars=25 μ m. PDL, periodontal ligament; R, dental root.

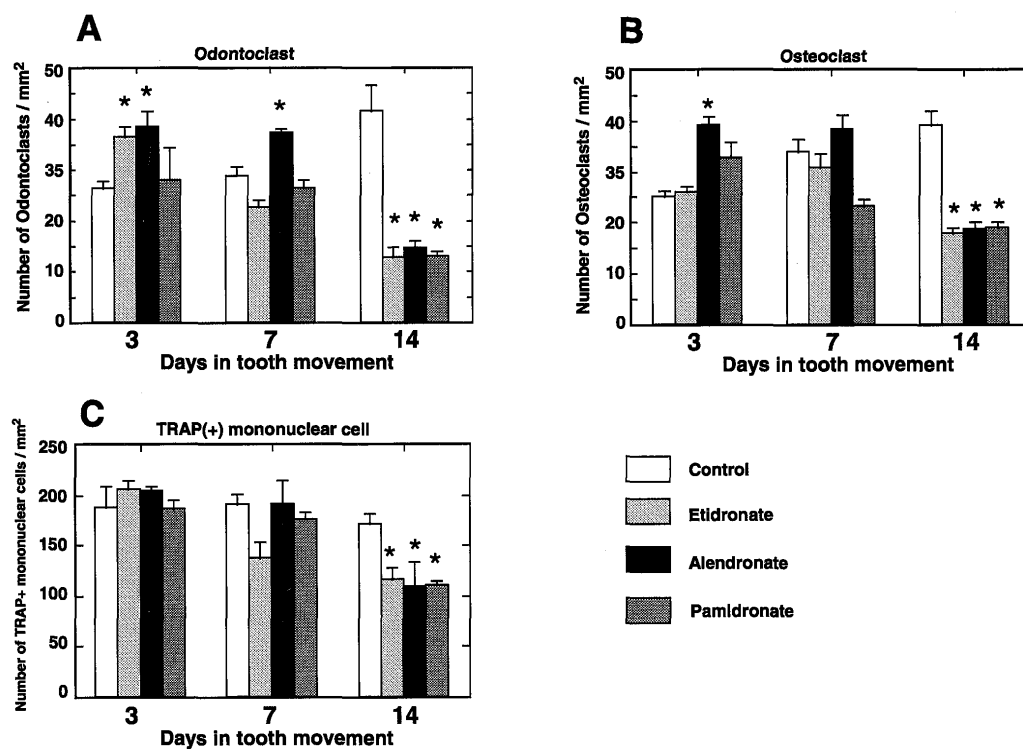


Fig. 6 Changes in number of odontoclasts (A), osteoclasts (B), and TRAP-positive mononuclear cells (C) in a rat dentoalveolar unit topically injected with 2.5 mg/kg of bisphosphonates during experimental tooth movement. The values from 5 representative sections of 5 or 6 animals each were averaged. Each column and bar represents the mean \pm standard error of the mean; * p <0.01 vs control group.

revealed the similar pattern with those in odontoclast numbers. A significant increase in the number of odontoclasts as well as osteoclasts was shown in etidronate- and alendronate-treated groups on day 3, but the number of precursors did not show a significant difference compared with the control group at that time.

3. Immunohistochemistry

To investigate the effect of topically applied bisphosphonates on cytokine expression, the immunohistochemical examination for IL-1 β , TNF- α , and IL-6 in the dentoalveolar unit was performed. In the control group, IL-1 β and TNF- α were expressed in odontoclasts, osteoclasts, and PDL cells on day 7, but the positive signals of both cytokines became weaker after 14 days (Figs. 7, 8). On day 3, treatment with etidronate and alendronate markedly enhanced the immunoreactivity for IL-1 β and TNF- α in odontoclasts, osteoclasts, and PDL cells compared with control groups. However, in pamidronate-treatment group, no visible differences in IL-1 β and TNF- α immunoreactivity were observed in these cells compared with the control groups (Figs. 7, 8). As shown in Fig. 9, the expression of IL-6 was not observed in the control and each bisphosphonate treatment group.

Discussion

Several reports have described the different factors involved in the control of bone resorption, but the information on the biological mechanisms of root resorption is still insufficient. Orthodontically induced root resorption is an unavoidable pathologic consequence of orthodontic tooth movement. If such root resorption could be prevented, it would be an important contribution toward reducing risk factors in orthodontic treatment. It has previously been reported that the experimental model by Waldo and Rothblatt¹⁵⁾ enables us to observe the process for not only bone remodeling^{15, 21-23)}, but also dental root resorption^{15, 23)} during tooth movement. Using this experimental model, we have shown for the first time that topical application of bisphosphonate -etidronate, alendronate, and pamidronate- is sufficient to lead to a reduction of mechanically induced root resorption in rat, based on qualitative histology and morphometric data. Upon consideration of the cell counts in parts of the periodontium, it became evident that the inhibition was effective for the tooth-resorbing odontoclasts, as well as for the bone-resorbing osteoclasts.

Bisphosphonates are known as inhibitors of osteoclastic bone resorption with high affinity for bone mineral and are currently used in the treatment of skeletal disorders such

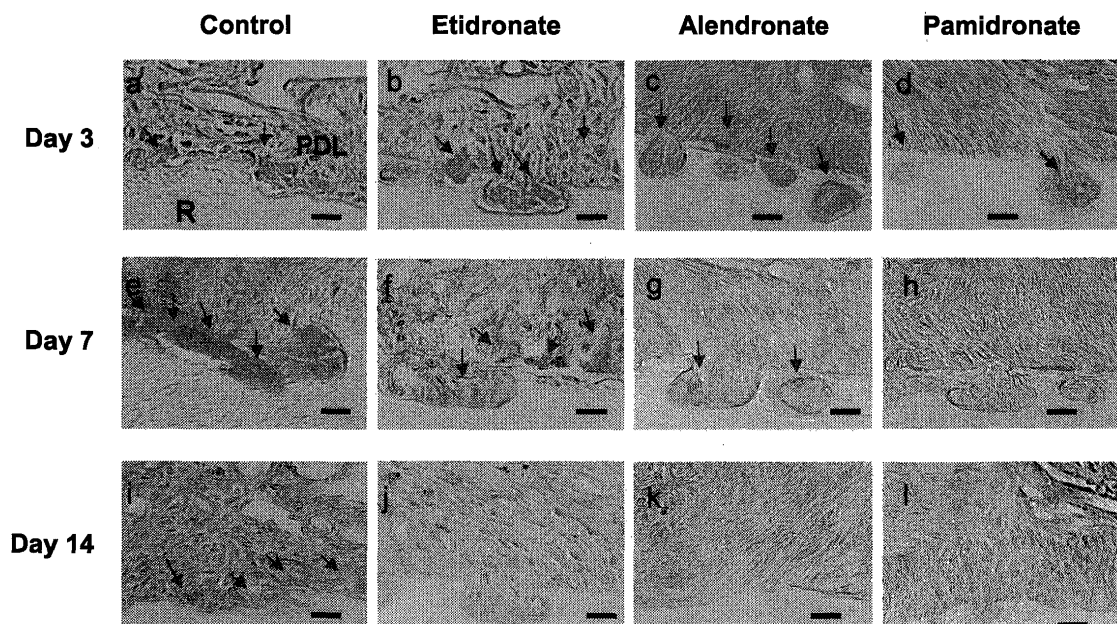


Fig. 7 Photomicrograph showing positive immunolabeling with anti-IL-1 β antibody in a rat dentoalveolar unit topically injected with 2.5 mg/kg of bisphosphonates - etidronate (b, f, j), alendronate (c, g, k), and pamidronate (d, h, i) - during experimental tooth movement. Control groups (a, e, i) received the same dose of saline vehicle. Tissues prepared at 3 (a - d), 7 (e - h), and 14 (i - l) days after insertion of the elastic band. The arrow indicates IL-1 β -positive cell on the root surface. Magnification 400 X. Bars=25 μ m. PDL, periodontal ligament; R, dental root.

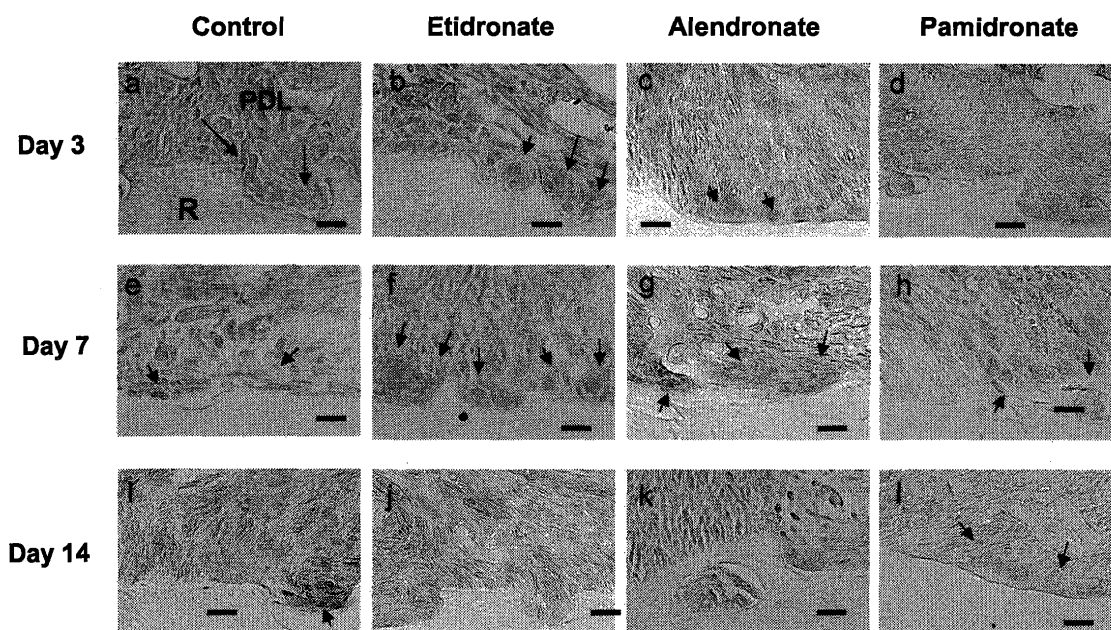


Fig. 8 Photomicrograph showing positive immunolabeling with anti-TNF- α antibody in a rat dentoalveolar unit topically injected with 2.5 mg/kg of bisphosphonates - etidronate (b, f, j), alendronate (c, g, k), and pamidronate (d, h, i) - during experimental tooth movement. Control groups (a, e, i) received the same dose of saline vehicle. Tissues prepared at 3 (a - d), 7 (e - h), and 14 (i - l) days after insertion of the elastic band. The arrow indicates TNF- α -positive cell on the root surface. Magnification 400 X. Bars=25 μ m. PDL, periodontal ligament; R, dental root.

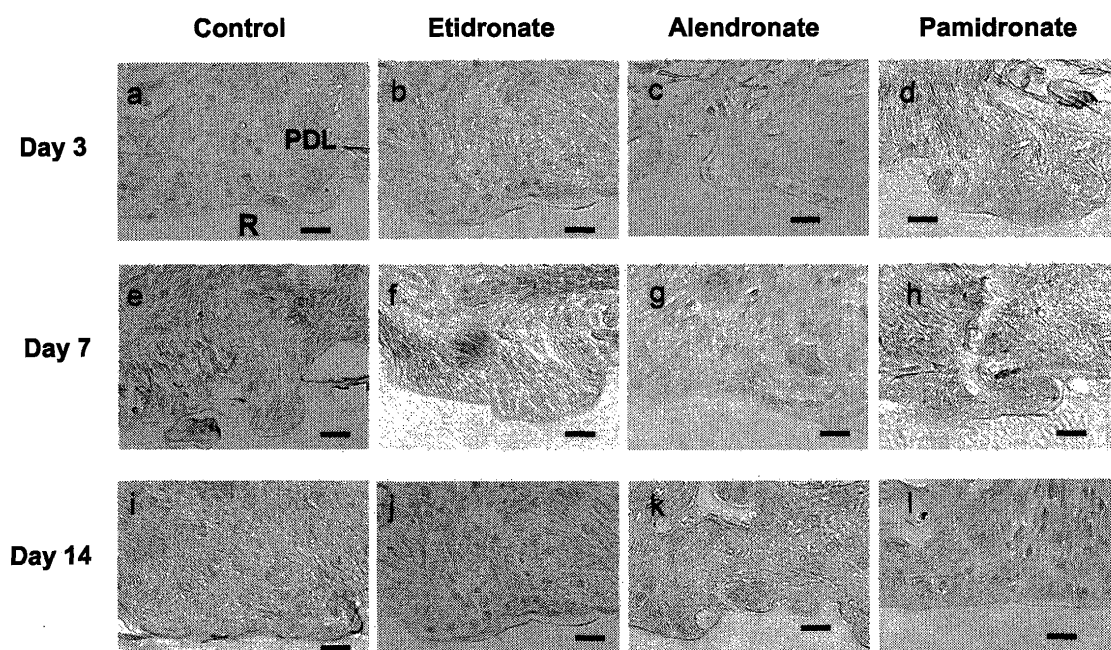


Fig. 9 Photomicrograph showing positive immunolabeling with anti-IL-6 antibody in a rat dentoalveolar unit topically injected with 2.5 mg/kg of bisphosphonates - etidronate (b, f, j), alendronate (c, g, k), and pamidronate (d, h, i) - during experimental tooth movement. Control groups (a, e, i) received the same dose of saline vehicle. Tissues prepared at 3 (a - d), 7 (e - h), and 14 (i - l) days after insertion of the elastic band. Magnification 400 X. Bars=25 μ m. PDL, periodontal ligament; R, dental root.

as osteoporosis²⁴⁾, Paget disease²⁵⁾, hypercalcemia²⁶⁾, and metastatic bone disease²⁷⁾. Non-N-containing bisphosphonates (e.g., etidronate) reduce osteoclastic bone resorption by producing toxic analogs of ATP that cause cell death¹³⁾. N-containing compounds (e.g., alendronate, pamidronate) interfere with protein prenylation by inhibiting farnesyl pyrophosphatase, an enzyme in the HMG-CoA reductase pathway⁶⁻¹²⁾. Inhibition of this enzyme prevents post-translational prenylation of guanosine triphosphatase (GTP)-binding proteins, which lead to reduce resorptive activity of osteoclasts and accelerated apoptosis. Direct inhibition of osteoclast activity is suggested by *in vivo* observations of the disappearance of ruffled border, which is convoluted the osteoclast membrane opposed to bone, associated with osteoclast activity²⁸⁾. With respect to inhibition of bone resorption in rats, alendronate and pamidronate have been evaluated to be 100-1000 times more potent than etidronate⁷⁾. As many investigations have clinically and pharmacologically proved that bisphosphonates possess different potencies to inhibition of bone resorption, to date, no clear-cut relationship between structure and activity could be perceived. In this study, the similar efficacy on inhibition of root resorption was revealed in etidronate, alendronate, and pamidronate at both of low (1 mg/kg) and high (2.5 mg/kg) doses, as well as on reduction of osteoclast formation. Since the number of TRAP-positive mononuclear cells, which includes odontoclast precursors, was reduced by bisphosphonate treatment, it is likely that formation and differentiation of odontoclasts, rather than their function, was inhibited by these bisphosphonates. Especially, these bisphosphonates are suggested to act at the early stage of odontoclast formation such as inducing proliferation of precursors. Studies on the inhibitory effect of odontoclastic root resorption by risedronate¹⁸⁾ that has a nitrogen molecule and clodronate²⁰⁾ that is a non-N-containing bisphosphonate have previously been reported. The present study is the first investigation to compare the capacity of the non-N- and N-containing bisphosphonates to inhibit odontoclastic root resorption, and the similar efficacy was demonstrated in these bisphosphonates. Therefore, these findings suggest that inhibition of odontoclastic root resorption might be universal in spite of the difference of their chemical structures.

Meanwhile, high dose treatment with etidronate and alendronate caused a transient enhancement of root resorption due to an increase in the number of odontoclasts, as well as that of osteoclasts. This event might be associated with cytokine production in response to bisphosphonates. Bisphosphonates, in particular the N-containing forms, are known to have a number of side-effects including a rise in body temperature and accompanying flu-like symptoms that

resemble a typical acute phase response²⁹⁾; these clinical features occur in over a third patients receiving treatment for the first time^{30,31)}. The mechanism for this response has been partially elucidated and appears to be associated with the release of TNF- α and IL-6³²⁻³⁴⁾, although the effector cells that release these cytokines and the cellular mechanism of action remain obscure. In addition, *in vitro* studies have demonstrated that bisphosphonates inhibit or accelerate the release of proinflammatory molecules such as IL-1 β ³⁵⁻³⁶⁾ nitric oxide³⁷⁾, prostaglandin E₂^{38,39)} in the monocyte-macrophage lineage cells. Current thinking has homed in on monocytes/macrophages as potential cellular sources of these proinflammatory agents³³⁾. It has been reported that proinflammatory cytokines such as IL-1 β ⁴⁰⁾, TNF- α ⁴¹⁾, and IL-6⁴²⁾ play important roles in bone remodeling *in vitro* and *in vivo*. Each of these cytokines has multiple activities, which include bone resorption and new bone formation. Exact mechanism of root resorption is not fully clarified, but two proinflammatory cytokines, IL-1 and TNF- α , have recently been shown to be important for the induction and the further process of mechanically induced root resorption⁴³⁾. In this study, it was immunohistochemically observed that IL-1 β and TNF- α production in odontoclasts, osteoclasts, and PDL cells was induced by etidronate and alendronate treatment at the initial stage, but not by pamidronate. This result may indicate that etidronate and alendronate exert the production of IL-1 β and TNF- α on odontoclasts, osteoclasts and PDL cells, and that the same intracellular pathways in these cells are responsible for production of IL-1 β and TNF- α stimulated by bisphosphonates. IL-6 has been thought to regulate immune response in inflammatory sites, and play an important role of osteoclast formation and differentiation. Previous studies⁴⁴⁻⁴⁶⁾ demonstrated that IL-6 production was induced in periodontal tissues during orthodontic tooth movement. In this study, however, the immunohistochemical analysis did not show IL-6 expression in response to mechanically induced tooth movement, and in addition IL-6 expression was not affected by all bisphosphonates. The action of both non-N-containing etidronate and N-containing alendronate led to production of IL-1 β and TNF- α in osteoclasts, odontoclasts, and PDL cells. The cellular mechanism of the proinflammatory cytokine activation caused by bisphosphonate structure is not well understood in these cells. Recently, nuclear factor-kB, a multifunctional transcriptional factor, has been thought to be associated with proinflammatory response⁴⁷⁾.

In clinical aspects, external root resorption is a frequent adverse effect of orthodontic tooth movement. Extensive apical root loss can impair successful treatment and, in certain situation, may decrease the functional capacity or the actual life of the tooth. Root resorption of permanent teeth after

transplantation is also an unsolved problem. Therefore, if root resorption can be prevented by chemical agents, this complex problem may be overcome. The present study is the first to evaluate the inhibitory effect of three bisphosphonates, which possess different chemical structure, on mechanically induced root resorption. Further studies are required to determine (1) the distribution of bisphosphonates on the root surface, (2) the dose that is necessary to inhibit odontoclast formation, without side-effects induced by cytokines, (3) the duration in which the administered bisphosphonates deposit on the root surfaces.

Conclusion

In this study, the efficacy of three bisphosphonates (etidronate, alendronate, and pamidronate) on inhibition of root resorption during experimental tooth movement was firstly determined. All bisphosphonates significantly inhibited root resorption which was induced by mechanical forces, in parallel with a significant reduction in the number of odontoclasts. On the other hand, a transient increase of root resorption by etidronate and alendronate treatment was revealed at the early stage, in accordance with IL-1 β and TNF- α production in odontoclasts, osteoclasts, and PDL cells. These results might be useful for future treatment regimes to prevent root resorption in the course of periodontitis, or during periodontal or orthodontic therapy.

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