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Long-term effects of local pretreatment with alendronate on healing of replanted rat teeth

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Background and Objective: Our previous study showed that topical alendronate, an inhibitor of bone resorption, reduces root resorption and ankylosis for 21 d after replantation of rat teeth. The aim of the present study was to evaluate the long-term inhibitory effects of topical alendronate in the replanted teeth.

Material and Methods: The rat maxillary first molars were extracted, placed in saline containing 1 mM alendronate (alendronate group) or saline (saline group) for 5 min and then replanted. The maxillae were dissected at 60 and 120 d. Microcomputed tomography horizontal sections at three root levels were analyzed for root and bone resorption, ankylosis and pulp mineralization.

Results: In the alendronate group at 60 and 120 d, the frequencies of resorption of roots and bone were lower than those in the saline group. The p values show statistical significances of lower frequencies in the alendronate group than in the saline group by chi-square test (see Table 1). Ankylosis and pulp mineralization occurred in the alendronate and saline groups. Bone marrow spaces were narrowed in conjunction with bone tissue expansion around the replanted teeth in the alendronate group.

Conclusion: The inhibitory effects of topical alendronate were retained on root and bone resorption, but not on ankylosis and pulp mineralization, in the replanted teeth for 4 mo. Alendronate might also stimulate bone formation around the rat replanted teeth.

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The clinical problems encountered in tooth replantation are resorption of the alveolar bones and roots, and ankylosis (1,2). It has been shown that topical pretreatment of root surfaces with alendronate, an inhibitor of osteoclast-mediated hard tissue resorption, reduces root loss, caused by resorption, after 4 mo in replanted dog teeth (3). We have further shown that pretreatment with alendronate reduces not only root resorption but also bone resorption, ankylosis and pulp mineralization in replanted rat teeth, leading to better restoration of the mechanical strength of the healing periodontal ligament to up to 67% of the normal control (4). In the latter study, these beneficial effects of alendronate were examined for only 21 d after treatment.

The biological half-life of alendronate, a bisphosphonate, has been reported to be at least 10 years in human bone following oral administration (5,6,7). Local administration of bisphosphonates is also effective and could avoid systemic side-effects. The affinity of bisphosphonates for calcium phosphate and their preferential binding to hydroxyapatite in bones and teeth lead to a rapid uptake into the calcified tissues (6). The deposited bisphosphonates are liberated again only when the tissues are resorbed. The half-life of bisphosphonates in such tissues could be long, dependent largely on the rate of turnover of the tissues (7). Thus, topical treatment with a bisphosphonate could be effective for a longer period of time (3).

The aim of the present study was to evaluate the long-term effects of local pretreatment with alendronate on the healing of replanted rat molars by analyzing microcomputed tomography images and histologic appearances at 60 and 120 d after tooth replantation.

Material and methods

The present study was approved by the Animal Care Committee of Tsurumi University School of Dental Medicine. Twenty-nine male Wistar rats, aged 34–36 d, were purchased from Nihon Clea (Tokyo, Japan). They were fed a powdered diet (CE-2; Nihon Clea) and given water *ad libitum* during the experimental period.

Replantation of rat maxillary first molar

The procedures of extraction and replantation of the maxillary first molar were described in detail previously (4). The rats (41–43 d of age; average body weight 170 ± 9 g) were anesthetized with intraperitoneal injections of ketamine hydrochloride (47 mg/kg of body weight) and medetomidine hydrochloride (0.4 mg/kg of body weight). Then, their left maxillary first molars were extracted, washed in saline (0.9% NaCl), placed in saline containing 1 mM alendronate (Teiroc® Inj.; Teijin, Osaka, Japan; alendronate group, $n = 13$) or saline (saline group, $n = 8$) for 5 min at room temperature of approximately 25°C, and then put back into their sockets. The rats were injected subcutaneously with benzylpenicillin procaine (2,000,000 U/kg of body weight) and awakened with an intraperitoneal injection of atipamezole hydrochloride (0.83 mg/kg of body weight). In normal control rats (normal control group, $n = 8$), the teeth were not extracted.

Microcomputed tomography

Under anesthesia with ketamine and medetomidine, the animals of the three groups were perfused with 0.1 M phosphate-buffered saline (pH 7.2), containing 4% paraformaldehyde, 60 and 120 d after tooth replantation. Thereafter, the maxillae were dissected and then placed in the same fixative at 4°C. Each maxilla containing a replanted tooth was horizontally scanned at 16-μm intervals with a microcomputed tomography system (MCT-CB100MFZ; Hitachi Medico, Tokyo, Japan), and 201 images were obtained (480 × 480 pixels). Three-dimensional images of the replanted teeth were reconstructed using software (TRI-3D; RATOC, Tokyo, Japan).

Image analysis

Horizontal images at cervical, middle and apical levels of the replanted teeth were reconstructed (Fig. 1) using reconstruction software (EXAVISION LITE; Ziosoft, Tokyo, Japan) as follows.

(i) A horizontal image of a replanted rat maxillary first molar was obtained approximately at the mid-region of the mesial and distal roots (Fig. 1B).
(ii) A mesio-distal longitudinal image of the replanted tooth (Fig. 1A) through an axis X (Fig. 1B) was obtained.
(iii) A reference line connecting the tips of the mesial and distal roots (RL) was drawn on the image (Fig. 1A). In parallel to the reference line, cervical, apical, and middle root levels were determined.
(iv) The cervical root level through the cementum surface of the furcation and the apical root level through the projecting site of the mesial surface of the mesial root in the apical region were defined.
(v) The middle root level was defined as occuring between the cervical and apical root levels.

In each horizontal image, mesial, lingual, linguo-distal and bucco-distal roots were divided into four parts by mesio-distal and lingo-buccal lines (Fig. 1B). For each part, we examined the presence or absence of: (i) ankylosis (direct fusion of bone to root; (ii) root resorption (resorption lacunae on the root surface; (iii) bone resorption (resorption lacunae on the alveolar bone surface); and (iv) pulp mineralization. Then, the number of parts with each symptom in each group was counted. The total number of analysis sites, except for pulp mineralization, in each group were four parts/root × four roots/tooth × three root levels/tooth × n rats. That of pulp mineralization in each group was four roots/

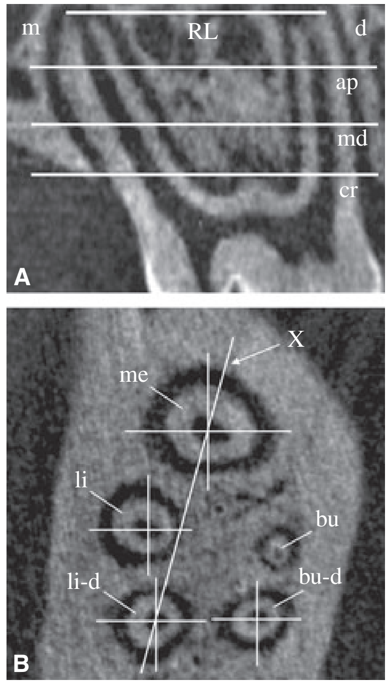

Fig. 1. (A) A mesio-distal, longitudinal image of a rat maxillary first molar through an axis *X* (B) reconstructed from microcomputed tomography. A reference line connecting the tips of the mesial and linguo-distal roots (RL) was constructed. In parallel to the RL line, the cervical root level through the cementum surface of the furcation and the apical root level through the projecting site of the mesial surface of the mesial root in the apical region were determined. The middle root level was determined as occuring between the cervical and the apical root levels. (B) A horizontal image of the same tooth at the middle root level. Each of the mesial, lingual, linguo-distal and bucco-distal roots was divided by mesio-distal and linguo-buccal lines into four parts for image analysis. The buccal root was not analyzed. ap, apical root level; bu, buccal root; bu-d, bucco-distal; cr, cervical root level; d, distal side; li, lingual root; li-d, linguo-distal root; m, mesial side; me, mesial root; md, middle root level.

tooth × three root levels/tooth × *n* rats. Mean frequencies (%) of sites with ankylosis, root resorption, bone resorption and pulp mineralization were calculated for each part, each root or each group. As the buccal root is thin (Fig. 1B) and sometimes missing, we did not analyze it. The chi-squared test was used to compare these categorical data between the alendronate and saline groups.

Histological examination

After the microcomputed tomography scanning, the maxillae were decalcified in 14.5% EDTA for 4 wk, dehydrated and embedded in paraffin. Mesio-distal longitudinal sections were cut serially with a microtome (Supercut 2050, Reichert-Jung, Heidelberg, Germany) setting of 6 μm, stained with hematoxylin and eosin and observed by light microscopy.

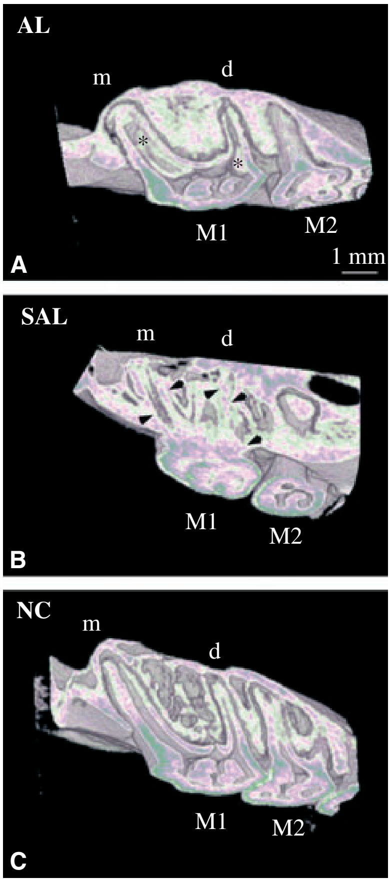

Fig. 2. Three-dimensional microcomputed tomography images of replanted rat maxillary first molars (M1) at 60 d (A,B) and a normal control tooth (C). The longitudinally sectioned surfaces were obtained by cutting three-dimensional reconstructed specimens through *X*-axes (Fig. 3A,C,E). A part of each tooth crown was also horizontally sectioned. The teeth were replanted after pretreatment with 1 mM alendronate (A) or saline (B) solution for 5 min. The normal control tooth in a rat of the equivalent age (C) was not treated. Asterisks indicate abnormal mineralization in the dental pulp. Arrowheads indicate ankylosis. d, distal side; m, mesial side; M2, second molar.

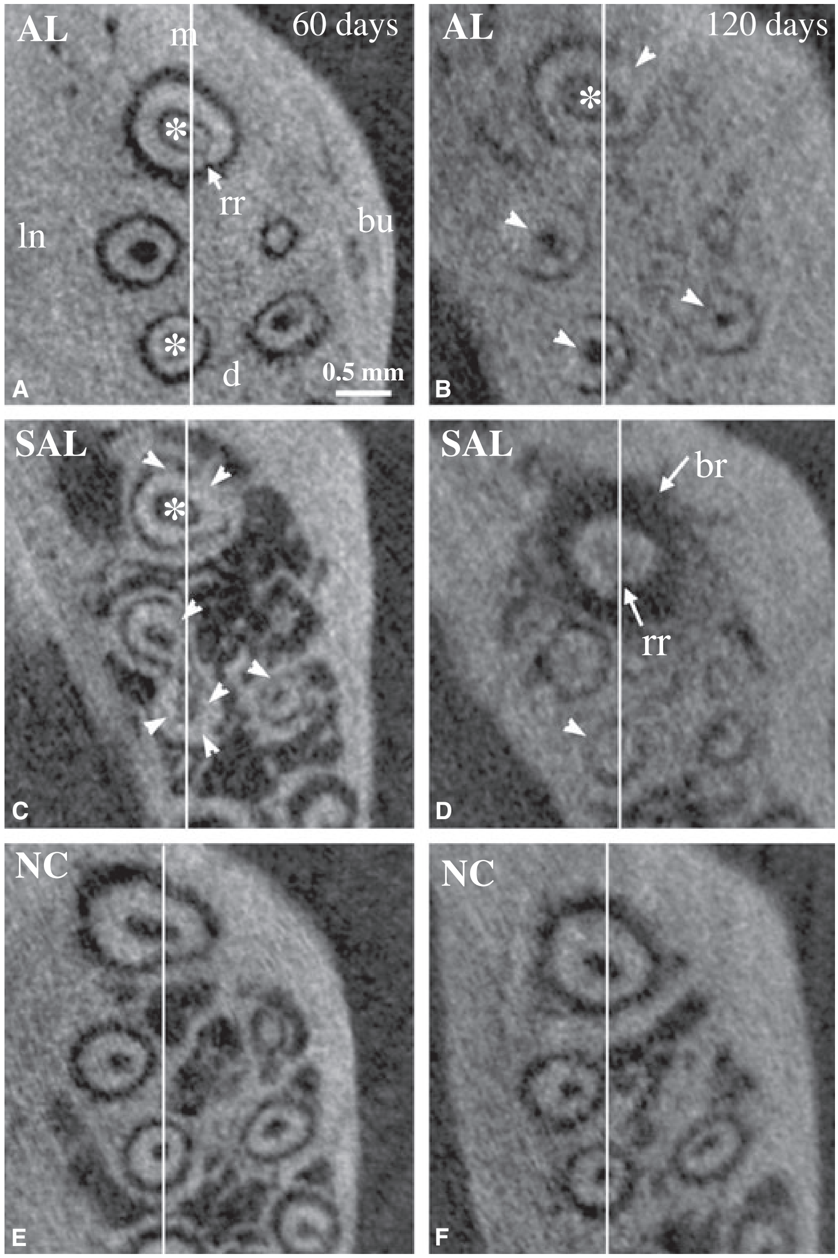

Fig. 3. Two-dimensional horizontal microcomputed tomography images of replanted rat maxillary first molars at 60 d (A,C) and 120 d (D,E) and normal control teeth (E,F) at the middle root level. The teeth were replanted after pretreatment with 1 mM alendronate (A,B) or saline (C,D) solution for 5 min. Asterisks indicate abnormal mineralization in the dental pulp. Arrowheads indicate ankylosis. AL, alendronate; br, bone resorption; bu, buccal side; d, distal; ln, lingual; m, mesial; NC, normal control; rr, root resorption; SAL, saline.

Results

Microcomputed tomography images of replanted teeth

The three-dimensional (Fig. 2) and two-dimensional (Fig. 3) microcomputed tomography images revealed the presence of periodontal spaces around most of the five roots in the alendronate-pretreated teeth at 60 d (Figs 2A and 3A), as in the normal control teeth (Figs 2C and 3E), but at 120 d the five roots of the teeth became ankylosed (Fig. 3B). The saline-pretreated teeth had several severe ankylotic areas around most of the five roots at 60 d (Figs 2B and 3C): at 120 d, severe resorption of roots and bone was also seen in some teeth (Fig. 3D). Abnormal mineralized structures were seen in the pulp of most of the teeth in both groups at 60 and 120 d. The alveolar bone surrounding the roots in the alendronate group at 60 d (Figs 2A and 3A) and at 120 d (Fig. 3B) had few bone marrow spaces, whereas the alveolar bone in the saline group had bone marrow spaces at 60 d (Figs 2B and 3C), as did the normal control teeth (Figs 2C and 3E).

Analyses of microcomputed tomography images

Figure 4 illustrates the averaged frequencies (color scale), for the three root levels, of sites with ankylosis, root resorption, bone resorption and pulp mineralization in diagrammatic horizontal sections representative of replanted teeth for the three groups.

The frequency of sites with ankylosis around the four roots did not show marked differences between the alendronate and saline groups at 60 and 120 d (Fig. 4): no significant differences in the averaged frequencies were found between the two groups at 60 and 120 d (Table 1). In the alendronate group, ankylosis around the four roots appeared to increase from 60 to 120 d (Fig. 4): the averaged frequency differed significantly between 60 and 120 d ($P < 0.001$, chi-square test; Table 1).

Root resorption and bone resorption around the four roots appeared to occur less frequently in the alendronate

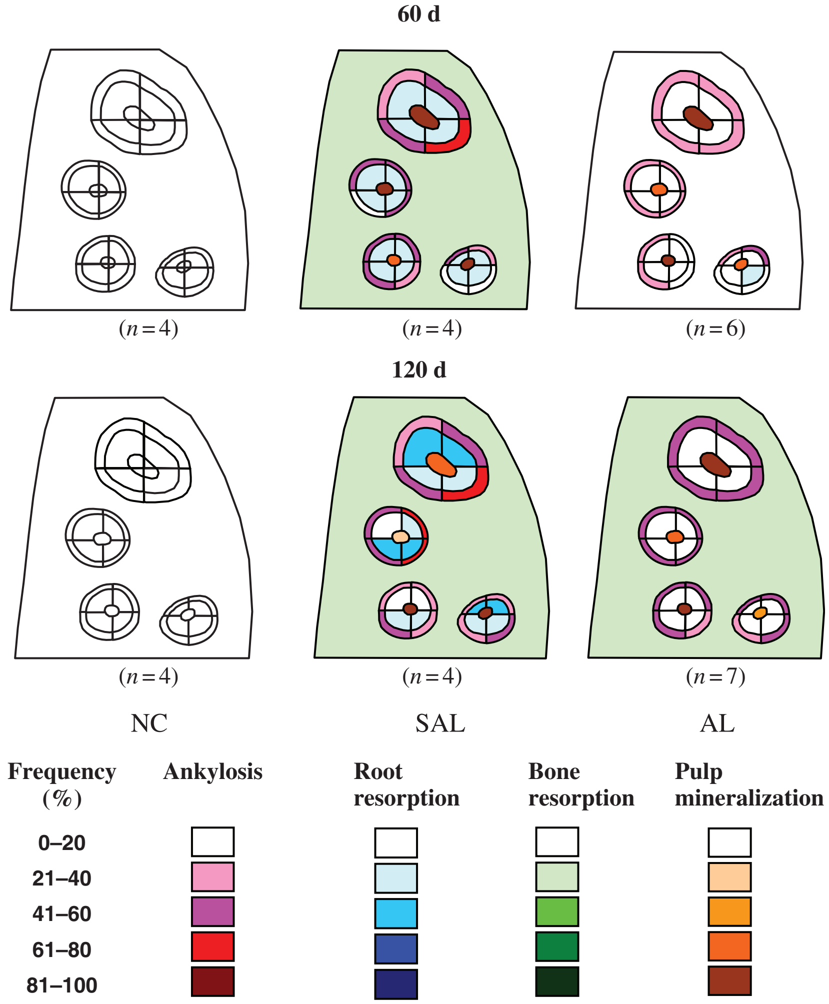

Fig. 4. Schematic representation of horizontal sections (Fig. 3) illustrating averaged frequencies (color scale) of ankylosis, resorption of roots and alveolar bone, and pulp mineralization in the alendronate, saline, and normal control groups at 60 and 120 d after tooth replantation. AL, alendronate; NC, normal control; SAL, saline.

Table 1. Percentage of sites with ankylosis, root resorption, bone resorption and pulp mineralization for the alendronate and saline groups at 60 and 120 d after tooth replantation

Days	Group	Ankylosis	Root resorption	Bone resorption	Pulp mineralization
60	Alendronate	28 (80/288)	3.5*** (10/288)	16* (47/288)	81 (58/72)
60	Saline	35 (51/144[a])	27 (51/192)	25 (48/192)	89 (32/36[a])
120	Alendronate	46### (155/336)	3.3*** (11/336)	22** (75/336)	70 (59/84)
120	Saline	42 (81/192)	32 (61/192)	34 (65/192)	77 (37/48)

Significant differences from the saline group, *, $p < 0.02$, **, $p < 0.01$, ***, $p < 0.001$ (chi-square test).
Significant differences from the alendronate group at 60 d, ###, $p < 0.001$ (chi-square test).
Data in parenthesis represent the number of sites with each symptom/total number of analyzed sites.
[a]In one rat of the saline group at 60 d, ankylosis and pulp mineralization could not be evaluated because of severe root resorption.

group compared with the saline group at 60 and 120 d (Fig. 4): the averaged frequencies of the alendronate group were significantly lower than those of the saline group at 60 and 120 d (P < 0.02–0.001, chi-square test; Table 1).

The frequencies of pulp mineralization in the four roots were similar in the alendronate and saline groups (Fig. 4): there were no differences in the averaged frequencies between the alendronate and saline groups at 60 and 120 d (Table 1).

Histological observation

Figure 5 shows light micrographs of mesio-distal longitudinal sections through replanted maxillary first molars pretreated with alendronate or saline, and age-matched normal control teeth. Both alendronate- and saline-pretreated teeth had ankylotic areas at 60 and 120 d (arrowheads, Fig. 5B–D). Bone-like nodules and blood cells were observed in the pulp of both alendronate- (Fig. 5A,B) and saline- (Fig. 5C,D) pretreated teeth. In the alendronate-pretreated teeth at 60 and 120 d, bone marrow spaces were narrowed in conjunction with bone tissue expansion around the mesial and distal roots (Fig. 5A,B) compared with the saline-pretreated and normal control teeth.

In magnified images of the periodontal ligament, the oblique collagen fibers were well restored near the alveolar crest at 60 and 120 d in the alendronate- (Fig. 6A) and saline- (Fig. 6C) pretreated teeth, as in the normal tooth (Fig. 6E). In the apical region, the oblique fibers were seen in the alendronate-pretreated teeth (Fig. 6B), as in the normal teeth (Fig. 6F). In contrast, only thin fibers parallel to the root surface were seen in the saline-pretreated teeth (Fig. 6D).

Discussion

The present study shows that topical treatment of roots with 1 mM alendronate for 5 min before tooth replantation effectively inhibited root and bone resorption for 120 d compared with the replanted teeth pretreated

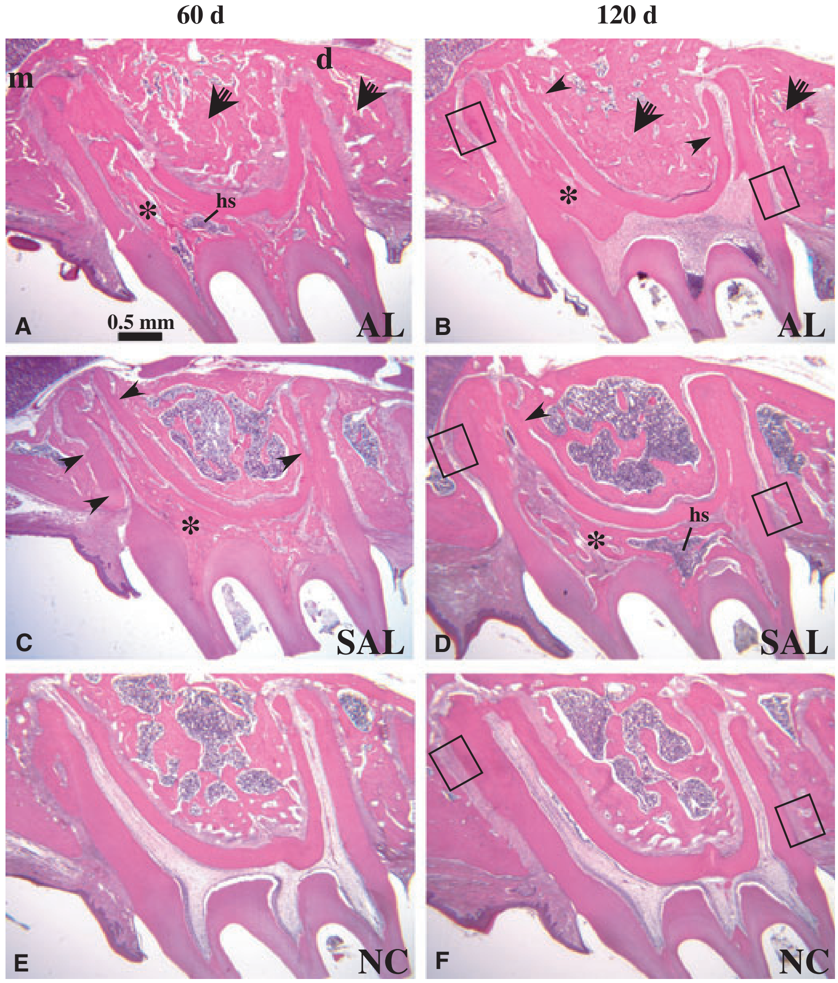

Fig. 5. Longitudinal histological sections of replanted rat maxillary first molars at 60 d (A,C,E) and 120 d (B,D,F). The teeth were replanted after pretreatment with 1 mM alendronate (A,B) or saline (C,D) for 5 min. Sections were also prepared from untreated teeth (E,F). Arrowheads indicate ankylosis. Arrows indicate narrowing of bone marrow spaces in conjunction with bone tissue expansion. Asterisks indicate bone-like tissues in the dental pulp. AL, alendronate; d, distal side; hs, hematoxophilic tissues in the dental pulp; m, mesial side; NC, normal control; SAL, saline. Hematoxylin and eosin staining; original magnification ×17.

with saline (Table 1). This is in agreement with a previous study on replanted dog teeth (3). These studies indicate that local treatment of tooth roots with alendronate before replantation could be useful for preventing bone and root resorption in replanted teeth. If the principal mode of action of alendronate is similar in human and rat replanted teeth, from the clinical point of view this finding is very important.

The affinity of bisphosphonates for calcium phosphate is high, and they preferentially bind to hydroxyapatite in bones and teeth (6). Topically administered alendronate would be rapidly deposited onto the adjacent calcified tissues. The bisphosphonates would be liberated again only when tissue remodeling occurred. The half-life of bisphosphonates in such tissues could be long, dependent largely on the rate of turnover of the tissues (6,7). As the turnover of cementum occurs only slightly (8), root surface-bound bisphosphonates are probably retained for longer than bone-bound bisphosphonates (9). Indeed, the frequencies of sites with root resorption were significantly lower (3.5 and 3.3%) than those of sites with bone resorption (16 and 22%) at 60 and 120 d after tooth

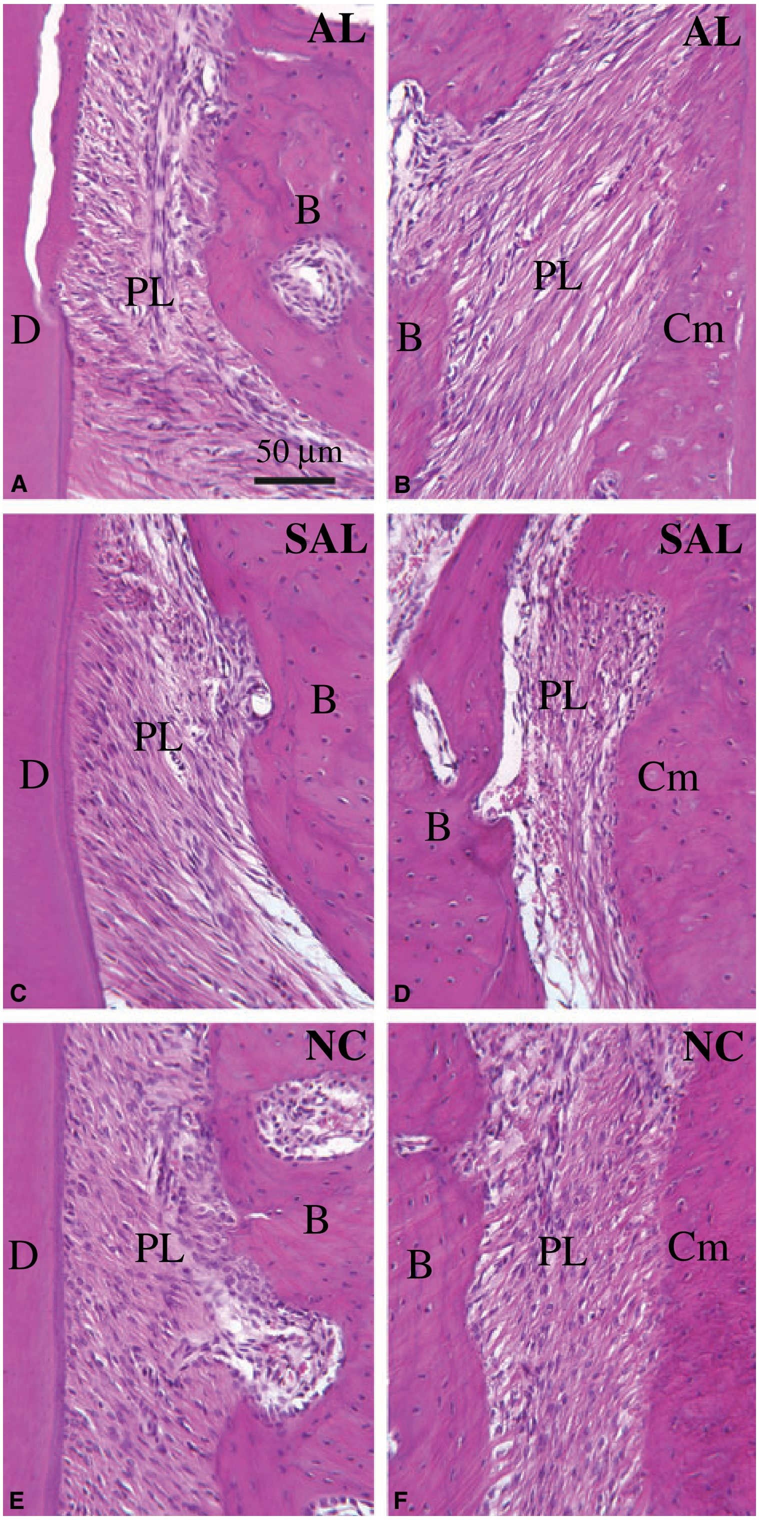

Fig. 6. Magnified views of the cervical regions (A,C,E) of the distal roots in Fig. 5 (boxed areas), and of the apical regions (B,D,F) of the mesial roots in Fig. 5 (boxed areas) of teeth replanted after pretreatment with alendronate (A,B) or saline (C,D), and of a normal tooth (E,F) 120 d after replantation. B, bone; Cm, cementum; D, dentin; PL, periodontal ligament. Original magnification ×230.

replantation in the alendronate group ($p < 0.001$, chi-square test; Table 1). It is suggested that the effective deposition of alendronate on the root and bone surfaces in the replanted teeth is retained for at least 4 mo.

The principal mode of action of alendronate is inhibition of osteoclast function. In particular, nitrogen-containing bisphosphonates, such as alendronate, are taken up by osteoclasts, where they inhibit an enzyme in the mevalonate pathway of cholesterol synthesis. This leads to reduction in the levels of geranylgeranyl diphosphate, which is required for GTP-binding proteins that are essential for osteoclast activity and survival (10). As the topical bisphosphonates inhibit not only bone resorption but also root resorption (3,4,9,11; Table 1), root surface-bound alendronate may also play a role in inhibiting the function of odontoclasts. It has been reported that alendronate inhibited bone resorption by isolated osteoclasts when the amount on the bone surface was around 1.3×10^{-3} fmol/μm^2, which would produce a concentration of 0.1–1 mM in the resorption space if 50% of the deposited alendronate was released (12). At a concentration of 1 mM, which we used in this study, alendronate may increase the leakiness of the ruffled border of osteoclasts to ions; bone resorption stops and the cells lose their ruffled border (12).

We have previously observed tendencies of less bone marrow and more bone tissues in the alendronate group at 14 and 21 d after tooth replantation (K. Komatsu *et al.* unpublished). In the present study, less bone marrow tissue and more bone tissue were more evident around the replanted teeth at 60 and 120 d in the alendronate group (Figs 2, 3 and 5). It has been reported that subcutaneous injections of clodronate (2 mg of P/d for 6 d) increased the bone-formation rate of tibia in rats fed a low-calcium diet (13). *In vitro* studies have shown that higher concentrations (10^{-6} to 10^{-4} M) of risedronate, alendronate and clodronate inhibited the formation of mineralized structures in rat bone marrow cell cultures (13,14). Lower concentrations (10^{-9} to 10^{-7} M) of risedronate and alendronate increased the total number of fibroblastic colonies without changes in mineralization (14). It has also been reported that a lower concentration (10^{-8} M) of alendronate, risedronate and zolendronate enhanced the proliferation of human trabecular bone cells, osteoblast-like cells (MG-63) and human bone marrow stromal cells (13,14), and stimulated the gene expression of bone morphogenetic protein-2, type I collagen, osteocalcin and/or core-binding factor alpha subunit 1 (15,16). Therefore, we assume that lower concentrations of alendronate released from surfaces of roots and alveolar bones on which it has been deposited may stimulate bone formation around the replanted teeth. Further studies should be necessary to investigate how alendronate affects the osteoblasts around the replanted teeth. This type of study is now in progress at our laboratory.

In the present study, the inhibitory effects of topical alendronate on the occurrence of ankylosis and pulp mineralization, as seen at 21 d (4), were no longer seen at 60 and 120 d. We assume that the inhibitory effects at 21 d may be caused by the physical-chemical inhibition of formation of calcium phosphate (6) and inhibition of the bone-forming activity of osteoblastic cells by relatively higher concentrations of alendronate deposited on the root and bone surfaces (13,14). At 60 and 120 d, more differentiated cells, stimulated by lower concentrations of the released alendronate (13,14), might have involved ankylosis and pulp mineralization.

The replanted teeth with several ankylotic areas tended to have a narrow periodontal ligament with only thin fibers parallel to the root surface in the apical and middle regions (Figs 5C,D and 6D). In contrast, the healing periodontal ligament with a few or no ankylotic areas in the alendronate group tended to have a greater width and functional arrangement of fiber bundles (Figs 5A,B and 6B). It may be possible that mechanical stimuli, such as occlusal contacts, play an important role in the regeneration of the periodontal ligament in replanted teeth (4,17,18). However, we have observed that in the cervical regions of the healing periodontal ligament, the supporting fibers with attachments to the cementum and bone surfaces were restored independently of ankylosis (Figs 5A–D and 6A,C). Geometrical locations of the periodontal ligament may affect the remodeling of those tissues after injury.

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