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Histological comparison of alendronate, calcium hydroxide and formocresol in amputated rat molar

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Abstract – The purpose of this study was to evaluate the potential of alendronate sodium (ALN), a biphosohonate to stimulate hard tissue formation in pulpotomized (amputated) rat molars. Two commonly used pulpotomy materials, calcium hydroxide (CH) and formocresol (FC) were utilized for comparisons. Histological evaluations were performed by observers blinded to treatment allocation on days 7, 15, 30 and 60, followed by statistical analysis of selected histological criteria. In all evaluation periods, hard tissue deposition was evident along the radicular dentin in ALN and CH groups. In days 30 and 60, the latter two groups showed no differences in inflammatory cell response and hard tissue deposition scores ($P > 0.05$). ALN appears to be capable of maintaining pulpal vitality, while promoting hard tissue formation, similar to CH.

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Key words: biphoshponates; alendronate; calcium hydroxide; formocresol; pulpotomy; pulp capping dental; adverse effects

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Pulpotomy is a common procedure in the management of young permanent teeth with open apices as well as the treatment of acutely inflamed primary teeth. Calcium hydroxide (CH), zinc oxide eugenol, gluteraldehyde, formocresol (FC), and more recently, collagen, freeze-dried bone and bone morphogenetic proteins have been proposed as pulpotomy agents (1–6). The use of electrosurgery and lasers has also been proposed for the pulpotomy procedure with an objective of preserving pulp vitality by minimizing damage to the radicular pulp tissue (5). FC, the most universally preferred and taught primary tooth pulpotomy medicament (1, 2), has been reevaluated over the years because of its toxic, mutagenic and carcinogenic potential to the host (3, 4). For several reasons, CH has met with acceptance for clinical use. Nevertheless, the material has been clinically observed to stimulate internal resorption, rather than reparative dentin formation in pulpotomized primary teeth (5). Studies have also shown that the use of CH can result in necrosis, acute or chronic inflammation, and dystrophic calcification in exposed pulp tissue (7–9).

Biphosphonates are carbon-substituted pyrophosphate analogues that include potent inhibitors of bone resorption. These therapeutics have recently been used effectively to control osteolysis or reduce bone loss in Paget's disease, metastastic bone disease, hypercalcemia of malignancy and osteoporosis (10). Alendronate sodium (ALN), a commonly-used biphosphonate, is known to favor hard tissue turnover and animal studies have shown that local administration of ALN ameliorates alveolar bone resorption after mucoperiosteal flap surgery (11, 12). Recent publications in the dental literature have extended the potential use of alendronate in tooth replantation, peri-implant defect regeneration, inhibition of bacteria-originated external root resorption, and apexification (13–16). To date, however, the direct effect of alendronate on

mechanically exposed pulp tissue has not been documented. The objective of the present study was to evaluate the effect of ALN on pulp tissue in comparison with CH and FC in amputated rat molars.

Materials and methods

Animals and surgical setup

Twenty-four locally-bred young (3–5 months old) Wistar albino rats weighing 270–300 g were used in this study. Only male rats were used in order to avoid effects of hormonal changes on hard tissue resorption mechanisms (12). The animals were cared for according to the policies and principles established by the Animal Welfare Act and the NIH Guide for Care and Use of Laboratory Animals (publication no. 86–23). During the entire test period, the animals were kept in rooms illuminated from 07:00 to 19:00 hours (12 h light/12 h dark cycle), maintained at 21–23°C, and had full access to pellet food and water *ad libitum*. All animals remained healthy throughout the duration of the study.

Animals were anesthetized with a mixture of ketamine hydrochloride (Ketalar, Parke-Davis, Pfizer Inc., NY, USA) and xylazine hydrochloride (Rompun 2%, Bayer, Dormagen, Germany) by intramuscular injection of 30 mg kg^{-1}. Access to the molars was obtained by the use of an operating table, providing visualization by aid of a binocular microscope.

Operative procedures

Class I cavities were prepared on first and second maxillary and first mandibular molars under copious sterile saline irrigation with an ISO 1/012 round diamond bur (Diatech, Bern, Sweden). Removal of the coronal pulp was performed with the help of a spoon excavator. Hemorrhage control was obtained by sterile cotton minipledgets. Pulpotomies were, then, performed with FC, ALN and CH. *FC group*: in all animals, left maxillary first and second molars were assigned for FC pulpotomies. Cotton pledgets moistened with full-strength FC (Buckley's Formula, Sultan Chemists, NJ, USA) were placed in contact with pulp stumps for 5 min. Thereafter, pulp stumps were covered with zinc oxide and eugenol cement and non-gamma 2 amalgam (Permite, SDI, Victoria, Australia). *ALN group*: sterile ALN powder (900 μg) was gently placed on the pulpal stumps of the right maxillary first and second molars. Care was taken to create a complete seal of the pulp stumps with ALN, while avoiding any pressure during placement of the material. The cavity was, then sealed with amalgam. *CH group*: calcium hydroxide pulpotomies were performed on the right and left mandibular first molars. Fresh CH paste was prepared by mixing pure CH powder and sterile distilled water. The paste was then placed on the pulp stumps without excessive pressure. The site was then covered with amalgam.

Histological evaluation

On the postoperative 7, 15, 30, 60 days, the animals were sacrificed with overdose ether inhalation anesthesia for histological evaluations. The maxilla and mandible were dissected free from the head and teeth were removed *en bloc* with surrounding bone and fixed immediately in 10% formic acid. Following a fixation period of 7 days, samples were decalcified in de Castro Fluid for 10 weeks. Thereafter, the teeth were washed, dehydrated, embedded in paraffin and sectioned serially at 5 μm in sagital direction. Sections were stained with hematoxylin and eosin and examined under a light microscope by two observers blinded to treatment allocation. Assessment of pulpal response was performed according to the following criteria (17):

1 Inflammatory cell response:

Score 0:
None or a few scattered inflammatory cells present in the pulp area corresponding to the pulp exposure, characteristic of normal tissue.

Score 1:
Slight inflammatory cell infiltrate with polymorph nuclear (PMNs) or mononuclear (MNLs) leukocytes.

Score 2:
Moderate inflammatory cell infiltrate involving the coronal third of the radicular pulp.

Score 3:
Severe inflammatory cell infiltrate involving the coronal third of the radicular pulp or characterizing abscess.

2 Tissue disorganization:

Score 0:
Normal tissue.

Score 1:
Slight disorganization near the exposure site with normal central pulp.

Score 2:
Moderate disorganization of pulp tissue.

Score 3:
Severe or total disorganization of pulp tissue.

Score 4:
Necrosis.

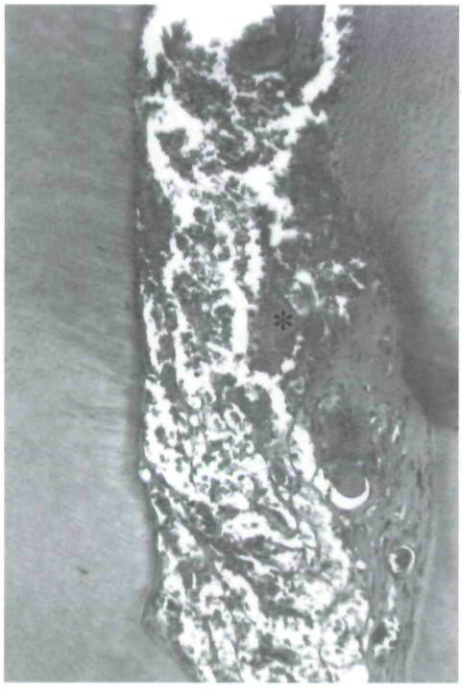

Fig. 1. A 15-day specimen in the FC group, showing hemorrhage and mononuclear cell infiltration. Homogenous eosinophilic zones (asterisk) can be seen within the fibrotic pulp. (H&E, ×20).

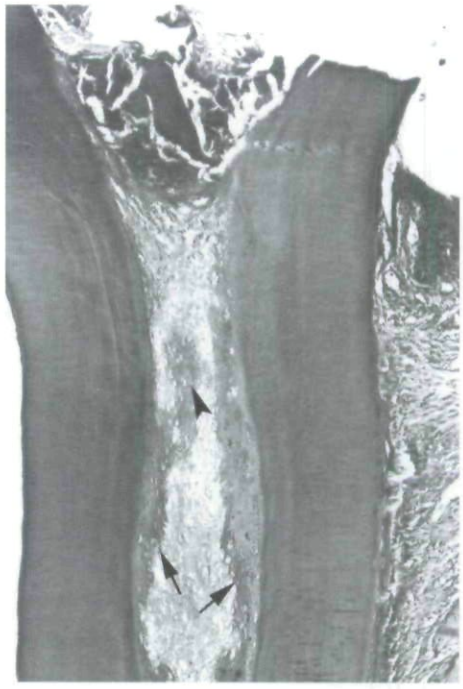

Fig. 2. Discrete dense fibrotic tissue underneath the exposure site in a 60-day FC specimen. A generalized fibrosis associated with eosiniphilic zones (arrowhead) and hyalinization (arrows) are evident. (H&E, ×10).

3 Hard tissue formation:

Score 0:
Absence.

Score 1:
Modest hard tissue deposition beneath or lateral to the exposed area.

Score 2:
Moderate hard tissue deposition beneath or lateral to the exposed area.

Score 3:
Complete dentin bridge formation beneath the exposed area.

In each section, histological criteria were evaluated for two separate segments: pulp tissue beneath exposure site (segment E), and the radicular pulp lateral to the exposure site, extending to the apex (segment R). Statistical analysis of time-dependent response for each material was performed using Kruskal–Wallis and Mann–Whitney *U*-tests ($P = 0.05$). A separate statistical evaluation was performed between ALN and CH scores using Mann–Whitney *U*-test at $P = 0.05$, as these two materials are expected to preserve vitality of the pulp.

Results

The surgical procedures were well-tolerated by the experimental animals. The postoperative period was uneventful, confirming the absence of any apparent adverse effect caused by the experimental protocol.

FC group

Moderate inflammation at the exposure site observed in early intervals (Fig. 1) was further replaced by a fibrous necrotic tissue in the long-term. Beneath this necrotic zone, the radicular pulp tissue showed a generalized disorganization and incomplete fixation. No dentin formation was observed. At day 60, the typical precursors of intracanal calcification (irregular hard tissue deposition and areas of hyalinization) were present in the apical part of pulp tissue (Fig. 2). Subadjacent to the exposure sites, a discrete dense fibrotic tissue was observed. One specimen showed abscess formation in the vicinity of root apices.

ALN group

At day 7, moderate inflammation and hemorrhage, characterized by a number of dilated blood vessels were present at the exposure site (Fig. 3). However, both were not persistent and resolved in the short-term. At day 30, dentin deposition started at the lateral walls of the root canal dentin and continued dramatically thereafter (Fig. 4). In the long-term (60 days), pulp tissue subjacent to the exposure site

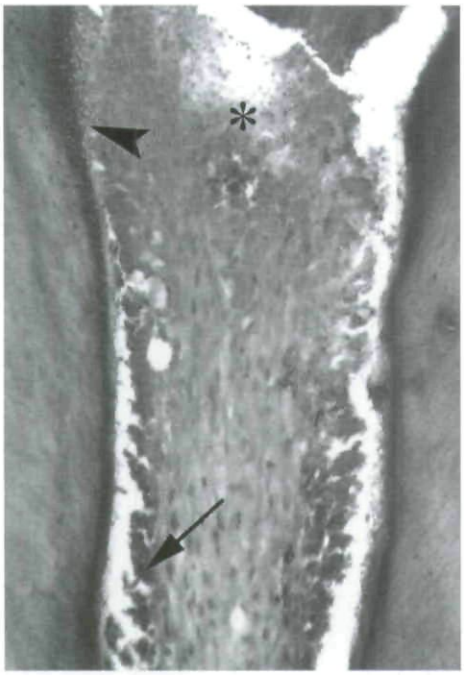

Fig. 3. A 7-day specimen in the ALN group, demonstrating hemorrhage at the exposure site (asterisk). An increase in odontoblastic activity (arrowhead) and slight mononuclear cell infiltration can be seen (H&E, ×20).

was more disorganized than the radicular pulp, showing discrete necrosis in the coronal third. No evidence of dentin matrix deposition was observed under the exposure site. However, continued dentin deposition was evident in the middle and apical dentinal walls (Fig. 5). The radicular pulp of the 60 day specimens exhibited fibrillar changes.

CH group

Light microscopic examination of the exposure site showed slight inflammation and hemorrhage in the short-term (Fig. 6). An increase in vascularization continued in the first 2 weeks. During this period, new dentin deposition was observed along the root canals, being slightly less than the ALN group. Thirty-day specimens showed a decrease in vascularization and mononuclear cell infiltration, with continuation of dentin deposition on the lateral walls. Similar to the ALN group, the radicular pulp of the 60 day specimens exhibited fibrillar changes. No dentinal bridging was observed beneath the exposure site. Nevertheless, continued deposition of radicular dentin matrix continued was evident (Fig. 7).

Statistical analyses of histological scores for each group are presented in Tables 1–3. In all groups, significant differences were found between the short-term and long-term groups in terms of inflammatory cell response and tissue disorganization ($P < 0.05$, Kruskal–Wallis and Mann–Whitney *U*-tests). Table 4 shows statistical comparison of histological data between the ALN and CH groups.

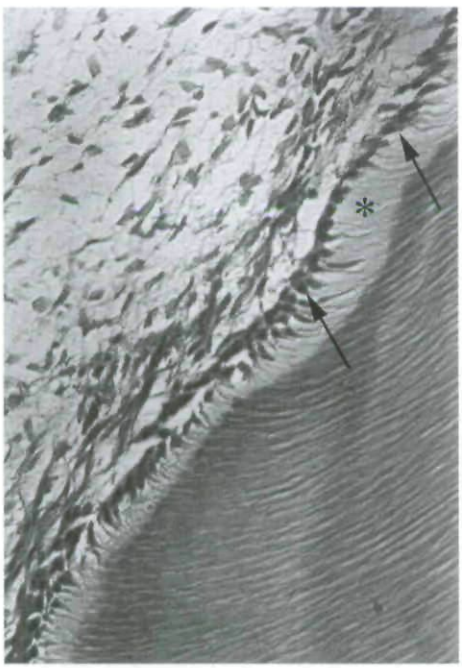

Fig. 4. Histological view of a 30-day ALN specimen demonstrating odontoblasts (arrows) and new dentin formation (asterisk). (H&E, ×40).

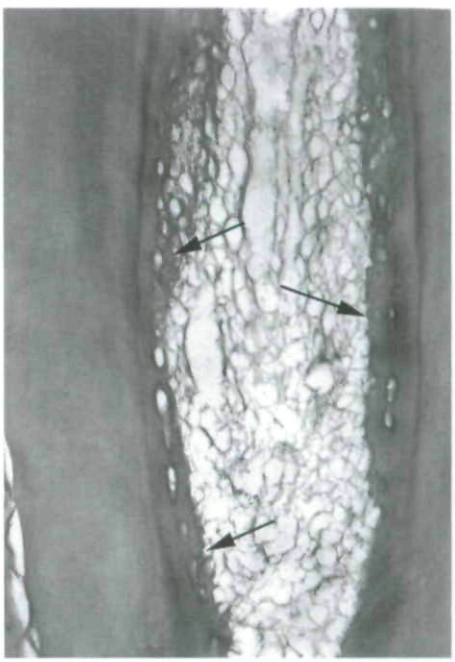

Fig. 5. Radicular pulp of a 60-day specimen in the ALN group. Remarkable dentin deposition is evident on lateral walls of the root canal (arrows), with no sign of internal resorption (H&E, ×20).

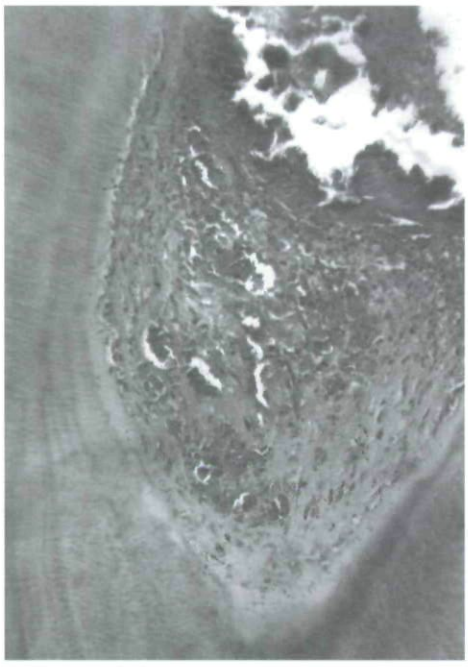

Fig. 6. A 15-day specimen in the CH group, showing hemorrhage subadjacent to the exposure site and increased vascularization. Dense mononuclear cell infiltration foci are evident, while there is an increase in odontoblastic activity. (H&E, ×20).

Accordingly, the inflammatory cell response and hard tissue formation scores at days 30 and 60 were not significantly different (Mann–Whitney *U*-test, $P > 0.05$).

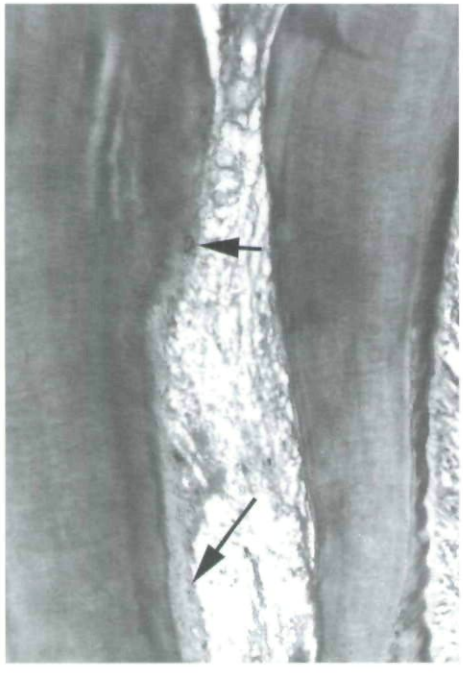

Fig. 7. Histological view of a 60-day CH specimen. Dentin deposition has taken place (arrow) on one side of the root canal, while there is no evidence of odontoblastic activity on the contra lateral wall (H&E, ×10).

Table 1. Statistical comparison of scores in the FC group

Evaluation period (day)	*n*	Mean	SD
INF E			
7	5	1.20[d,f]	0.45
15	5	3.00	0.00
30	5	1.40[d]	0.55
60	4	0.25[f]	0.50
Total	19	1.53	1.07
INF R			
7	5	0.40[a,e,g]	0.89
15	5	0.00[a,j]	0.00
30	5	1.40[e]	0.55
60	4	0.25[g,j]	0.50
Total	19	0.53	0.77
Disorganization E			
7	5	0.00	0.00
15	5	1.80[h,k]	0.45
3	5	2.20[h,m]	0.45
60	4	2.00[k,m]	0.00
Total	19	1.47	0.96
Disorganization R			
7	5	0.00	0.00
15	5	1.60[l,l]	0.89
30	5	2.20[l,n]	0.45
60	4	2.00[l,n]	0.00
Total	19	1.42	1.02
Hard tissue E			
7	5	0.00[b]	0.00
15	5	0.00[b]	0.00
30	5	1.00[o]	0.71
60	4	1.00[o]	0.00
Total	19	0.47	0.61
Hard tissue R			
7	5	0.00[c]	0.00
15	5	0.00[c]	0.00
30	5	1.00[p]	0.71
60	4	1.00[p]	0.00
Total	19	0.47	0.61

Groups identified with same lettering designate scores for each event that are not significantly different at $P = 0.05$.
INF, inflammatory cell response; Disorganization, tissue disorganization; Hard tissue, Hard tissue formation; E, pulp tissue just beneath exposure site; R, radicular pulp lateral to the exposure site extending to apex.

Discussion

In the pulpotomy model, the ratio of pulp surface area in contact with capping material to the remaining pulp tissue is considerably higher in comparison with a direct pulp-capping model. A material with a high potential to cause side-effects, is more likely to elicit problems in narrow pulp canals than in the wider coronal pulp (6). For this reason, pulpotomy seems to be an advantageous model for testing new materials, as it allows them to exhibit their primary effect on the tissue as well as their possible undesirable effects, such as internal resorption, intracanal calcification or necrosis (5, 6).

While the rationale for the frequent use of FC is unclear, it is known to fix affected and infected radicular pulp tissue through mummification. This allows a chronic inflammation to be replaced with

Table 2. Statistical comparison of scores in the ALN group

Evaluation period (day)	*n*	Mean	SD
INF E			
7	6	1.67[a,e]	0.52
15	5	1.20[a,g]	0.45
30	6	1.50[e,g]	0.55
60	5	0.20	0.45
Total	22	1.18	0.73
INF R			
7	6	1.17[b,f]	0.41
15	5	1.20[b,h]	0.45
30	6	1.50[f,h]	0.55
60	5	0.20	0.45
Total	22	1.05	0.65
Disorganization E			
7	6	1.00	0.00
15	5	2.00[i]	0.00
3	6	2.33[i]	0.52
60	5	3.60	0.55
Total	22	2.18	1.01
Disorganization R			
7	6	0.00	0.00
15	5	2.00[j]	0.00
30	6	2.33[j,k]	0.52
60	5	3.00[k]	0.00
Total	22	1.77	1.19
Hard tissue E			
7	6	0.00[c]	0.00
15	5	0.20[c]	0.45
30	6	1.00[l]	0.00
60	5	1.60[l]	0.55
Total	22	0.68	0.72
Hard tissue R			
7	6	0.00[d]	0.00
15	5	0.00[d]	0.00
30	6	1.00[m]	0.00
60	5	1.60[m]	0.55
Total	22	0.64	0.73

Groups identified with same lettering designate scores for each event that are not significantly different at $P = 0.05$.
INF, inflammatory cell response; Disorganization, tissue disorganization; Hard tissue, hard tissue formation; E, pulp tissue just beneath exposure site; R, radicular pulp lateral to the exposure site extending to apex.

Table 3. Statistical comparison of scores in the CH group

Evaluation period (day)	*n*	Mean	SD
INF E			
7	6	0.83[b,g]	0.41
15	6	2.50	0.55
30	5	1.00[b]	0.00
60	5	0.20[g]	0.45
Total	22	1.18	0.96
INF R			
7	6	0.83[c,h]	0.41
15	6	2.50	0.55
30	5	1.00[c]	0.00
60	5	0.20[h]	0.45
Total	22	1.18	0.96
Disorganization E			
7	6	0.00	0.00
15	6	1.33[i,m]	0.52
3	5	1.00[i]	0.00
60	5	2.20[m]	0.45
Total	22	1.09	0.87
Disorganization R			
7	6	0.00[a,d]	0.00
15	6	0.67[a,j,n]	1.03
30	5	0.00[d,j]	0.00
60	5	2.20[n]	0.45
Total	22	0.68	1.04
Hard tissue E			
7	6	0.17[e]	0.41
15	6	1.00[k,o]	0.00
30	5	0.80[e,k,q]	0.45
60	5	1.40[o,q]	0.55
Total	22	0.82	0.59
Hard tissue R			
7	6	0.17[f]	0.41
15	6	1.00[l,p]	0.00
30	5	0.80[f,l,q]	0.45
60	5	1.40[p,q]	0.55
Total	22	0.82	0.59

Groups identified with same lettering designate scores for each event that are not significantly different at $P = 0.05$.
INF, inflammatory cell response; Disorganization, tissue disorganization; Hard tissue, hard tissue formation; E, pulp tissue just beneath exposure site; R, radicular pulp lateral to the exposure site extending to apex.

Table 4. Statistical comparison of scores between the ALN and CH groups

	INF E	INF R	Disorganization E	Disorganization R	Hard tissue E	Hard tissue R
7-day	0.041	0.093*	0.002	1.000*	0.699*	0.699*
15-day	0.009	0.009	0.082*	0.082*	0.030	0.030
30-day	0.177*	0.177*	0.004	0.004	0.662*	0.662*
60-day	1.000*	1.000*	0.016	0.032	0.690*	0.151*

*Mean scores of observed criteria that are not significantly different at $P = 0.05$.

an acute one, enabling the pulp tissue to remain in a metastable condition until the tooth exfoliates (6). Although the technique has a high clinical success rate, histological and radiographic success rates fail to support this data (18–20). In the present study, FC was selected as the negative control group. The unfavorable tissue response confirms previous studies, demonstrating chronic inflammation in the residual pulp tissue with virtually no sign of healing. Clearly, these findings explain the radiographic pathologies observed in symptom-free FC pulpotomized teeth, which have been judged successful by clinicians. Root canal obliteration, other than internal resorption and necrosis, is one of the frequent complications of FC pulpotomies (18, 19). Similarly, formation of irregular dentin in the 60th day samples herein was a similar sign of such pathological oblite-

ration, rather than a reparative activity of pulp tissue (20).

The hemosiderin mass detected in the majority of short-term histological specimens (7 and 15 days) demonstrated postoperative hemorrhage beneath the exposure sites, despite excellent hemostasis achieved before placement of test materials. Success in the pulpotomy procedure strongly depends on absolute control of hemorrhage, regardless of the material used in contact with pulp tissue (18, 21). It is known that hemosiderin delays or prevents recovery of the pulp (21). For ethical reasons, pulp capping studies are primarily conducted in animal models and the material placement is performed under general anesthesia, providing little if any effect in the controlling pulpal blood flow (22). Contrary to dental anesthesia in humans involving the use of local anesthetics with vasoconstrictors, pulpal hemorrhage may reoccur in the postoperative period, even if successful hemostasis is achieved under general anesthesia (22). Thus, in the present study, postoperative hemorrhage was considered to be one possible factor contributing to the delay of pulpal healing at the pulpotomy sites. Nevertheless, healing in the radicular segments in both ALN and CH groups was not influenced by postoperative hemorrhage. It should also be noted that delayed healing along the pulpal wound could be an expected protective response from the organism to any foreign material.

A marked deposition of reparative dentin along the root canal in the ALN group is indicative of a possible mechanism by which direct administration of ALN on amputated rat pulp may stimulate reparative dentin formation along radicular dentin. This finding may be explained in part by two studies demonstrating that alendronate supports osteoblast-mediated bone formation (23, 24). Odontoblasts share similar characteristics with osteoblasts, and the effect of ALN in accelerating dentin formation by odontoblasts has been recently shown by Sommercorn et al. (25) *in vitro*. In their study, the concentrations used to stimulate osteoblasts to form new bone also stimulated odontoblasts to form new dentin. The rate of dentin deposition was slower for the first 30 days when compared with the second 30 days (25), corroborating with the histological results obtained within the present study.

In the present study, histological evidence has been collected revealing that ALN may have the potential to induce odontoblast activity, leading to dentin formation without emanating any adverse effects in amputated rat molars. Further research is indicated to evaluate the ideal dose/concentration for stimulation of odontoblasts, before use of ALN as an alternative vital pulpotomy material can be advocated.

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