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The effects of topical application of bisphosphonates on replanted rat molars

Sung Chul Choi[1], Yong-Dae Kwon[2], Kwang Chul Kim[3], Gue-Tae Kim[4],

[1]Department of Pediatric Dentistry and Institute of Oral Biology, School of Dentistry, Kyung Hee University; [2]Department of Oral and Maxillofacial Surgery and Institute of Oral Biology, School of Dentistry, Kyung Hee University; [3]Department of Pediatric Dentistry, East West Neo Medical Center, School of Dentistry, Kyung Hee University; [4]Department of Oral and Maxillofacial Radiology and Institute of Oral Biology, School of Dentistry, Kyung Hee University, Seoul, Korea

Correspondence to: Kwang Chul Kim, DMD, MSD, PhD, Department of Pediatric Dentistry, East West Neo Medical Center, School of Dentistry, Kyung Hee University, #149 Sangil-dong Gangdong-gu, Seoul, 134-727, Korea
Tel.: +82-2-440-6207
Fax: +82-2-440-7549
e-mail: juniordent@khu.ac.kr

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Abstract – The purpose of this study was to evaluate the potential usefulness of two bisphosphonates (BPs) (etidronate and zoledronate), compared with that of alendronate, which is a well-known drug for delayed replantation, in decreasing or preventing inflammatory root resorption and replacement root resorption in replanted teeth. Eighty-four Sprague Dawley rat maxillary first molars were extracted, dried for 60 min and then replanted after root treatment. The rats were divided into four groups (control, alendronate, etidronate, zoledronate) as following treatments of avulsed root before replantation. At 7, 14, and 28 days postreplantation, the animals were sacrificed and the samples obtained and process for microscopic analysis. The data were statistically analyzed with the SPSS procedure, ANOVA test and each test was complemented by the Tukey's *post hoc* test. The result indicated that topical application of alendronate and zoledronate, both nitrogen containing BPs, prevented inflammatory root resorption and inflammatory cell response in the delayed replantation model. Both drugs were demonstrated similar effects in the delayed tooth replantation model ($P = 0.9$). Etidronate did not prevent inflammatory root resorption and inflammation in the delayed replantation ($P < 0.05$). No significant differences in replacement root resorption were observed among all drugs. These results suggest that when teeth are dried and not replanted immediately, zoledronate, like alendronate, may prevent root resorption and facilitates the regeneration of periodontal tissues after replantation.

The best clinical protocol of an avulsed tooth is immediate tooth replantation (1). When the avulsed tooth is immediately replanted and the vitality of periodontal fibers attached to the root is preserved, the chances of successful replantation increase considerably (2, 3). The best prognosis of tooth replantation is obtained when the extra-alveolar time does not exceed 5 min (4). However, it may be impossible for a patient and guardian who lack specific dental knowledge to immediately replant an avulsed tooth. Therefore, according to each condition, several supportive procedures may be utilized at the time of replantation through dental service, and the prognosis of replantation and the periodontal tissue healing response may vary (5, 6).

Studies investigating delayed tooth replantation are aimed at preventing inflammatory root resorption or reducing inflammation to save the time of regeneration and reattachment of periodontal ligament cells (7–10). Application of 2% acidulated phosphate sodium fluoride solution for 20 min is recommended by the International Association of Dental Traumatology (IADT) guidelines for delayed tooth replantation (11).

Bisphosphonates (BPs) are widely used drugs for patients with bone disorders showing increased osteoclast activity, such as Paget's disease (12, 13), hypercalcemia of malignancy (14), and osteoporosis (15). Depending on the content of BPs in molecular level, BPs can be divided into non-nitrogen containing BPs, such as clodronate, etidronate, tiludronate and nitrogen-containing BPs, such as alendronate, pamidronate, risedronate, and zoledronate (16). Recently, nitrogen containing BPs were further divided into alkyl-amino BPs (alendronate and pamidronate) and heterocyclic N-BPs (risedronate and zoledronate) (17, 18).

There have been many studies investigating the use of BPs during dental trauma mainly focusing on the use of alendronate and etidronate by means of topical root treatment and intracanal agents (8, 19, 20). Although the effects of alendronate as intracanal agent are in debate (20, 21), topical application of alendronate has been shown to decrease root apex inflammation and inhibited root resorption in delayed replanted animal teeth (8, 19, 22).

Among BPs currently in use, zoledronate, which is most frequently administered intravenously, has the highest binding affinity with Hydroxyapatite. Moreover, zoledronate is characterized by the most rapid onset and longest lasting action of all BPs and as such constitutes a

promising candidate for use in dental applications (23–25). Under the assumption that zoledronate pharmacological effects on the inflammatory inhibition are superior to etidronate and the alendronate, we tested the treatment effects by setting the control group as the negative control and alendronate group as the positive control.

The purpose of this study was to evaluate the usefulness of two kinds of BPs (Etidronate and Zoledronate), compared with that of alendronate which is well known to decrease or prevent inflammatory root resorption in delayed replantation of the rat teeth.

Material and method

Preparing animals and surgical procedures

This research proposal was reviewed and approved by the Ethics in Institutional Animal Care and Use Committee of Kyung Hee Medical Center, Kyung Hee University, Seoul, Korea (KHMC-IACUC: 09-004). Eighty-four, 5- to 6-week old, male Sprague Dawley rats (Semtaco, Kyungkido, Korea) weighing 200–250 g were used in this study. The rats were divided into four groups according to the root treatment method after extra-alveolar 60-min dry, as follows: Group I – no root treatment prior to replantation; Group II – 10-min soaking in 1 mM alendronate solution (Sigma-Aldrich Inc., St. Louis, MO, USA) in Hanks' Balanced Salt Solution (HBSS) and replanted; Group III – 10-min soaking in 20 mM etidronate solution (Sigma-Aldrich Inc.) in HBSS and replanted; and Group IV – 10-min soaking in 1.48 μM zoledronate solution (Novatis Pharma AG., Basel, Switzerland) in HBSS before replantation. All groups were divided into three subgroups according to the sacrificed day for histological examination: sacrificed at 7, 14, and 28 days post-treatment.

In this study, we questioned whether an avulsed tooth soaked in the optimal dose of zoledronate and etidronate would prevent resorption in replantation *in vivo*. Recent investigators reported that inflammatory root resorption in delayed replanted teeth could be decreased by soaking the avulsed tooth in 1 mM alendronate solution (8, 10, 26). To determine the optimal dose of etidronate and zoledronate for an *in vivo* study, we calculated the same ratio of drugs for the treatment of osteoporosis in humans comparing with alendronate (etidronate: 200 mg per day, alendronate: 70 mg per week, zoledronate: 5 mg per year). Therefore, we chose to soak the avulsed teeth in 20 mM etidronate and 1.48 μM zoledronate in HBSS.

For all procedures, animals were anesthetized with an intramuscular injection of Zoletil 50 (100–150 mg kg^{-1}; Virbac Lab, Carros, France). They were given a 5-day supply of 0.4% β-aminopropionitrile (β-APN; Sigma-Aldrich Inc.) to avoid traumatic extraction. Then, a clean operational field was obtained with a 2% chlorohexidine solution and the left maxillary first molars were atraumatically extracted using extraction forceps, and the molars were then placed in a pink wax sheet to dry at room temperature.

To minimize pulpal infection as stimulus for external root resorption, all teeth were accessed, instrumented with K-type files to the apical stop, and irrigated with sterile saline. Canals were dried with paper points and obturated with calcium hydroxide paste (Metapaste; Meta Biomed Co., Chungbuk, Korea). Occlusal accesses were filled with Caviton (GC Co., Tokyo, Japan). In all groups, teeth were dried at room temperature (20–24°C) for 60 min, and treated root canal during dry time. After drying at room temperature, the root was placed in citric acid (pH 1) for 3 min, curetted and cleaned with saline for 2 min to remove impaired and dead periodontal tissues, and then the teeth were soaked in the relevant medium. All avulsed sockets were cleaned with enough saline prior to replantation. After replantation, the rats were subcutaneously injected with gentamicin (0.1 ml kg^{-1}: Daesung Microbiological Lab, Kyungkido, Korea) and supplied with a soft diet for 7 days. Two dead rats, eight rats with fractured root, and left two rats were excluded to reach six animals in each group.

Histological evaluation and statistical analysis

On postoperative days 7, 14, and 28 days, all animals were sacrificed by means of flowing fixation with 10% formalin under anesthesia with Zoletil 50. The relevant maxilla was dissected free from the head and samples were fixed in 10% buffered formalin for 2 weeks. Following fixation, samples were decalcified in 0.1 M EDTA for 4 weeks. Thereafter, the maxillae were washed, dehydrated, embedded in paraffin, and sectioned serially at 5–8 μm in the saggital orientation by microtome. Sections were stained with hematoxylin and eosin and examined under a light microscope by seven dentists who were blinded to the treatment methods. Assessment of histological examination was performed as shown in Table 1.

We used the scores from five dentists who had received histological instruction for statistical analysis. Root resorption, inflammation and ankylosis were measured for the three types of BPs at the three times of sacrifice. We used SPSS for Windows Evaluation Version Release 15.0.0 (September 6, 2006) to evaluate treatment effects according to sacrifice day. First, we estimated with the parameters of the SPSS general linear model univariate procedure, two-way ANOVA or non-parametric

Table 1. Categories for scoring the statues of estimated point

Score	Inflammatory root resorption	Replacement root resorption	Inflammatory cell infiltration
0	None	None	None
1	Mild: cementum only	Mild: under 1/3 of the root area	Mild
2	Moderate: cementum and dentin resorption under 1/2 of the root area	Moderate: between 1/3 and 2/3 of the root area	Moderate
3	Sever: cementum and dentin resorption over 1/2 of the root area	Severe: over 2/3 of the root area	Severe
4	Tooth perforation		
5	Complete root resorption		

methods, and the Friedman test with 5% significance level. Each test was complemented by the Tukey's *post hoc* test. When this method revealed interaction between any of the primary factors, one-way ANOVA with Tukey's *post hoc* tests were employed to detect statistically significant differences between groups.

Result

Inflammatory root resorption

As time increased, the root resorption score increased in all groups ($P < 0.05$). But the root resorption scores were significantly different according to the type of BPs ($P < 0.05$, Table 2). In group I, mean value of inflammatory root resorption increased from 1.77 at 7 days up to 3.57 at 28 days. Especially, the tendency of root resorption was substantially high, cementum and dentin were absorbed in most roots and perforation was observed at some roots at 15 days. Most control groups at 30 days revealed severe root resorption including dentin, with some subjects having no remaining root. Among BP groups, the group III exhibited similar levels of inflammatory root resorption ($P < 0.05$, Table 2). But group II showed the lowest root resorption rate. As time points increased, the root resorption increased mildly, but the cementum and dentin were observed not to be absorbed to a greater degree (mean values 1.47 at 7 days to 2.17 at 28 days) (Table 2). The group IV was statistically equivalent to the group II ($P = 0.9$, Table 2).

Replacement root resorption

Replacement root resorption from delayed tooth replantation was not significantly different according to sacrifice time with 5% level. All experimental groups show similar degree of replacement resorption (Table 3).

Inflammatory cell response

In inflammatory cell response, ANOVA with Tukey's *post hoc* analysis demonstrated that the group III showed most severe inflammatory cell response. The inflammatory cell responses were differed significantly according to the sacrifice time. As time increased, the inflammatory responses tended to increase. Comparing the BPs according to the time increased by one way ANOVA with Tukey's *post hoc* analysis, group III showed a significant difference at 7 days from group II and IV and at 30 days from group II ($P < 0.05$). In the 14 days from all groups, there were no statistical differences (Table 4).

Table 2. Histological assessed mean values (±SD) of inflammatory root resorption according to the drugs and sacrificed days

	Inflammatory root resorption			
Experimental group		7 days	14 days	28 days
Group I	1	1.77 ± 0.63[a]	3.30 ± 0.70[b]	3.57 ± 0.73[d]
Group II	2	1.47 ± 0.57[a]	1.80 ± 0.71[c]	2.17 ± 0.83[e]
Group III	1	1.77 ± 0.63[a]	2.67 ± 1.15[bc]	3.27 ± 0.87[d]
Group IV	2	1.47 ± 0.51[a]	1.77 ± 0.50[c]	2.43 ± 0.50[e]

1 and 2 is significant difference in two-way ANOVA test ($P < 0.05$), different letters indicate statistical significant in one way ANOVA test comparing each group according to the time increased ($P < 0.05$).

Table 3. Histological assessed mean values (±SD) of replacement root resorption according to the drugs and sacrificed days

	Replacement root resorption		
Experimental group	7 days	14 days	28 days
Group I	0.23 ± 0.50	0.30 ± 0.47	0.13 ± 0.35
Group II	0.50 ± 0.63	0.30 ± 0.50	0.20 ± 0.50
Group III	0.53 ± 0.63	0.23 ± 0.45	0.10 ± 0.31
Group IV	0.20 ± 0.46	0.23 ± 0.50	0.10 ± 0.40

In replacement resorption, the incidence is too low to estimate statistical differences.

Table 4. Histological assessed mean values (±SD) of inflammatory cell response according to the drugs and sacrificed days

	Inflammatory cell response			
Experimental group		7 days	14 days	28 days
Group I	1	1.33 ± 0.48[a]	1.50 ± 0.51[c]	2.67 ± 0.48[de]
Group II	1	1.17 ± 0.65[a]	1.33 ± 0.48[c]	1.67 ± 0.48[e]
Group III	2	2.17 ± 0.68[b]	2.00 ± 0.59[c]	2.67 ± 0.48[d]
Group IV	1	1.67 ± 0.89[a]	1.37 ± 0.49[c]	2.17 ± 0.59[de]

1 and 2 is significant difference in two-way ANOVA test ($P < 0.05$), different letters indicate statistical significant in one way ANOVA test comparing each group according to the time increased ($P < 0.05$).

Discussion

It is essential to evaluate the effects of therapeutic drugs on delayed tooth replantation of avulsed teeth in experimental animals, such as dogs and monkeys, before any clinical application of the drug for human (7, 8, 27). The rat molar model employed in the present study can serve, because the procedures of extraction and replantation of rat molars are simple and easy (19, 22, 26, 28). This model allows for examination of the various aspects of the healing process in a sufficient number of animals to provide some statistical power to our findings.

To atraumatically extract the teeth, the animals were given a 5-day supply of β-aminopropionitrile which inhibits cross-linking of collagen in the periodontal ligament fiber and is reversed by drug cessation (29). In replantation studies, endodontic treatment can reduce experimental error by means of inhibition of inflammatory cells spreading from root canal to the apex and root surface. Therefore we used the calcium hydroxide for endodontic treatment. Polson and Proye (30) reported that citric acid treatment of denuded root surfaces resulted in new connective tissue attachment, so we followed the same procedure using a 3-min citric acid soaking before tooth replantation.

In this animal study of delayed replantation, topical treatment of roots with 1 mM alendronate for 10 min before replantation produced significant inhibition of inflammatory root resorption at 28 days compared with other experimental groups (Table 2). Our results were in agreement with those of the previous studies on delayed replantation that the use of alendronate inhibited inflammation, stimulated bone formation, and gave a better chance in normal healing of periodontal ligament in delayed tooth replantation (8, 10, 19).

Moreover, topical treatment with 1.48 μM zoledronate for 10 min before replantation effectively inhibited root resorption at 28 days. The inhibition effect of zoledronate is similar to that of alendronate (Table 2). The effects of zoledronate in delayed tooth replantation are not known, but in the allograft remodeling model, zoledronate can, just as previous shown with alendronate (31) decrease the bone resorption by systemic or soaking method (25, 32). Our hypothesis is that effect of zoledronate would last longer than that of alendronate; however, this was not borne out in the current study. Zoledronate is not superior to alendronate in this regard, yet alendronate is also not significantly better than zoledronate. These two drugs, nitrogen containing BPs, are effective and showed similar effects in this delayed tooth replantation model. However, etidronate is a commonly used drug, but did not inhibit inflammatory cells in the current study.

In this study, normal healing process after localized inflammatory root resorption, reinsertion of the periodontal ligament and new bone formation were observed around replanted teeth at 7, 14, and 28 days in alendronate group (Fig. 1). It has been reported that soaking in alendronate prevented inflammatory root resorption and stimulated bone formation in replanted rat teeth (10, 19). In zoledronate group, localized inflammatory root resorptions were observed frequently, but periodontal ligaments were normally reinserted to cementum and severe root resorption did not observed. Inflammatory reactions with multinucleated giant cells were more severe in zoledronate group than those in alendronate group (Fig. 2).

In this study, we questioned whether an avulsed tooth soaked in these dosages of drugs would prevent inflammatory root resorption in replantation in vivo. The bioavailability after systemic administration is irrelevant to that after topical application. It is possible that the results would be very different if different concentrations of BPs had been used, for example, the dosage of alendronate had reached their maximum effect, whereas the used concentrations of etidronate and zoledronate were very low and its effect could be optimized with a multiple concentration of the one used. Either a sound basis for choice of the dosages should be provided or a range of dosages should be tried.

Our data suggest that when avulsed tooth is not replanted immediately and delayed in a dried condition, zoledronate, like alendronate, may prevent root resorption and allow for time to regeneration of periodontal tissues. Further study regarding the optimal dosage of zoledronate to use in tooth replantation and the development of topical drugs as alternatives to injection drug would likely prove beneficial for dentists and patients.

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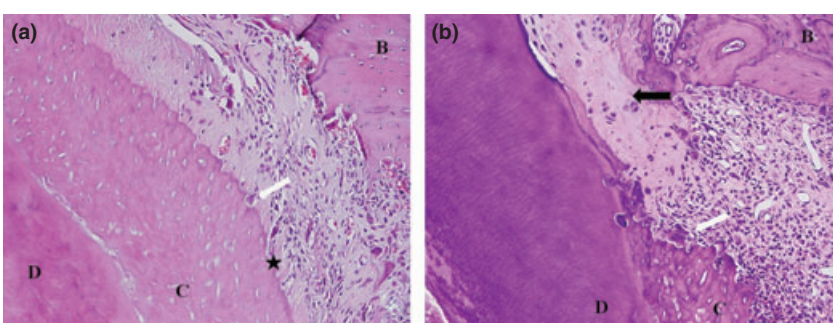

Fig. 1. Alendronate group, (a) 7 days. Photomicrograph showing reinsertion of periodontal ligament (black star) and multinucleated cells (white arrows) are observed in restricted area. (b) 30 days. Photomicrograph showing inflammatory root resorption (white arrow), new bone formation (black arrow) (B alveolar bone, C: cementum, D: dentin, H&E × 20).

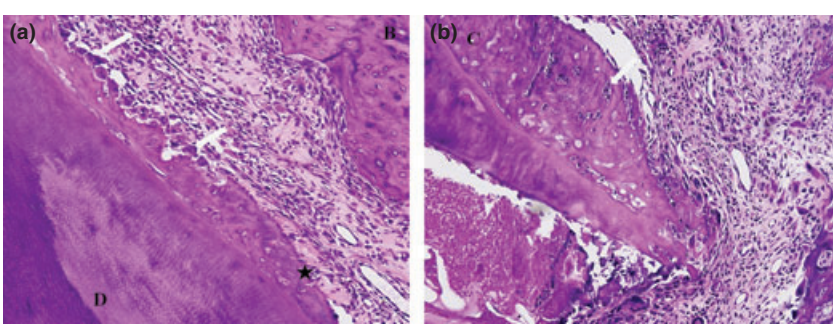

Fig. 2. Zoledronate group, (a) 7 days. Photomicrograph shows that root surface is undergoing inflammatory root resorption with multinucleated cells (white arrows) in cementum area and periodontal ligament is reinserted into cementum (black star). (b) 30 days. Photomicrograph showing localized inflammatory root resorption (white arrow) (B alveolar bone, C: cementum, D: dentin, H&E × 20).

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