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Chemical removal of necrotic periodontal ligament on delayed replanted teeth by sodium hypochlorite: morphological analysis and microhardness indentation test of cementum

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Abstract

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Aim To compare the efficacy of sodium hypochlorite (NaOCl) used at different concentrations and working times for removing necrotic periodontal ligament (PDL) from delayed replanted teeth and to observe the effects of NaOCl on surface structure and microhardness of cementum.

Methodology A total of 88 healthy premolars with a single root extracted for orthodontic purposes were selected and kept dry at room temperature for 1 h. The teeth were divided into 11 groups: group 1 (control): roots were untreated; group 2: necrotic PDL was removed with gauze; groups 3–11: teeth were immersed in NaOCl at different concentrations (1, 2.5 and 5.25%) and for different working times (5, 10 and 15 min). The specimens in each group were inspected separately for cementum integrity and the presence of PDL remnants by histomorphometric analysis, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Another 14 healthy premolars with roots divided into two pieces were selected for Vickers microhardness indentation tests before and after NaOCl treatment. The data were analysed statistically using Wilcoxon signed-rank test of two-related samples ($P = 0.05$).

Results In teeth treated with 1% NaOCl for 15 min or 5.25% NaOCl for 5 min, the cementum remained morphologically intact without cracks, and PDL remnants were absent. In the 1% NaOCl for 15 min group, the microstructure of cementum was arranged more regularly, as observed ×8000 magnification by SEM. Teeth in each of the other groups displayed cementum damage and/or the presence of PDL remnants. Microhardness tests revealed that treatment with 1% NaOCl for 15 min or 5.25% NaOCl for 5 min significantly decreased microhardness of root cementum ($P < 0.05$).

Conclusions Use of either 1% NaOCl for 15 min or 5.25% NaOCl for 5 min was effective at removing necrotic PDL from the delayed replanted teeth whilst having a minimal influence on cementum integrity. However, 1% NaOCl for 15 min was less damaging to cementum.

Keywords: cementum, microhardness, periodontal ligament, replanted tooth, scanning electron microscopy, sodium hypochlorite (NaOCl).

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Introduction

The prognosis of a replanted tooth relies on several factors, including the extra-alveolar period, desiccation of the root surface and the media in which the avulsed

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tooth is stored before replantation (Andreasen *et al.* 1995a,b). If the avulsed tooth is not immediately replanted or is not stored in an appropriate medium, this invariably leads to necrosis of the periodontal ligament (PDL) (Andreasen *et al.* 1995b). The presence of necrotic PDL remnants may then trigger external root resorption (Hammarstrom *et al.* 1986, 1989, Andreasen *et al.* 1995b, Hupp *et al.* 1998, Pohl *et al.* 2005, Panzarini *et al.* 2008), which is the major cause of replanted tooth loss (Panzarini *et al.* 2008). Therefore, in cases of delayed replantation, proper treatment of the root surface is necessary to increase the survival rate of the replanted tooth. Several authors have suggested the removal of necrotic PDL fibres as the first step of root surface treatment (Trope 2002, Panzarini *et al.* 2005, 2008). Other authors instead describe the application of fluoride solution, doxycycline, tetracycline, propolis or other substances directly on the necrotic PDL, without first removing the necrotic fibres; however, ankylosis and replacement resorption were noted following use of this method (Cvek *et al.* 1990, Selvig *et al.* 1992, McDonald & Strassler 1999, Panzarini *et al.* 2014).

Removal of necrotic PDL remnants is currently recommended as an attempt to increase the survival rate of the replanted tooth (Andersson *et al.* 2012). However, during this removal, efforts should also be taken to protect the cementum layer as it is more resistant to inflammatory resorption than dentine (Lindskog *et al.* 1985, Hammarstrom *et al.* 1989, Wallace & Vergona 1990, Lindskog & Blomlof 1992, Panzarini *et al.* 2008).

Several techniques have been used to remove necrotic PDL remnants prior to delayed replantation, including mechanical removal by scraping the root surface with curettes (Nyman *et al.* 1985, Zervas *et al.* 1991), scalpel blades (Duggal *et al.* 1994), polishing with a pumice/water slurry using rubber cups (Kenny *et al.* 2000) or bristle brushes (Esper *et al.* 2007), diamond burs or sandpaper dicks (Ripamonti & Petit 1989, Selvig *et al.* 1990). However, each of these methods left visible PDL remnants on the root, and the integrity of the cementum layer was largely destroyed.

Because of its capacity for dissolving connective tissue and its bactericidal effects, various concentrations of sodium hypochlorite (NaOCl) have been used for necrotic PDL removal in animal studies (Lindskog *et al.* 1985, Panzarini *et al.* 2005, Lustosa-Pereira *et al.* 2006, Sottovia *et al.* 2006). However, the proper combination of concentration and working time for NaOCl application for PDL removal and its effects on the cementum layer are unknown.

The purpose of this study was to compare the efficacy of NaOCl at different concentrations and different working times on removing necrotic PDL of delayed replanted teeth. In addition, the effect of NaOCl on surface structure and microhardness of cementum was assessed.

Materials and methods

Eighty-eight healthy premolars with a single root that were extracted for orthodontic purposes from adolescents of 14–16 years of age were included. The study was approved by the Ethics Committee of Peking University, and the parents of all patients gave their written informed consent for use of the teeth. After extraction, the teeth were immediately fixed to a sheet of red wax (Changzhi Glister Dental Equipment Corp. Ltd, Shanxi, China) with the crown facing down, set aside to dry at room temperature (26–28 °C) for 60 min, and then rehydrated by immersion in 50 mL saline (Beijing Double-Crane Pharmaceutical Equipment Corp. Ltd, Beijing, China) for 10 min. The teeth were then examined at ×80 magnification under a dissecting microscope to identify and eliminate any teeth with cracks or hypoplastic defects. As no teeth were eliminated, the original 88 premolars were then divided into the following 11 groups ($n = 8$):

Group 1 (control): the teeth were untreated.

Group 2 (gauze wipe): attached, nonviable soft tissue on the root surface was removed in a crown-apex direction with gauze which was dipped in saline.

Groups 3–11: the junctional epithelium and PDL fibres close to the cemento-enamel junction (CEJ) were detached from the root surface with wet gauze, and then the teeth were immersed in 20 mL NaOCl (Preparation Department of Peking University School and Hospital of Stomatology, Beijing, China) at different concentrations (1, 2.5 and 5.25%) and for different working times (5, 10 and 15 min) at room temperature (Table 1). After treatment with NaOCl, the teeth were rinsed with saline for 30 s and then immersed in 50 mL saline for an additional 10 min.

All the crowns were removed at the CEJ using a high-speed diamond bur (EX-41; Mani, Inc., Utsunomiya, Tochigi, Japan) under filtered water cooling.

From each group, three roots were used for histomorphometric analysis, three roots were observed

Table 1 Treatment groups with different concentrations of NaOCl and different working times ($n = 72$)

NaOCl concentration	1%	2.5%	5.25%
Working time (min)			
5	Group 3	Group 6	Group 9
10	Group 4	Group 7	Group 10
15	Group 5	Group 8	Group 11

under confocal laser scanning microscopy (CLSM) (LSM 5 PASCAL; Zeiss, Jena, Germany) using a fluorescence staining technique, and the other two roots were used for scanning electron microscopy examination (S-4800; Hitachi, Tokyo, Japan).

Histomorphometric analysis

The three roots selected for histomorphometric analysis were fixed in 10% neutral-buffered formalin (Yili Fine Chemicals Corp. Ltd, Beijing, China) for 24 h and then decalcified in 10% EDTA solution (Merck KGaA, Darmstadt, Germany) for 6–8 months. After decalcification, two roots were sectioned longitudinally in half, and one root was sectioned transversally, each in the middle third area of the tooth, using a diamond bur under filtered water cooling; the fragments were embedded in paraffin. Serial sections of 6-μm thickness were cut and stained with haematoxylin–eosin (HE) (Zhongshan Golden Bridge Biotechnology Corp. Ltd, Beijing, China). All samples were observed by a system microscope (BX51; Olympus, Tokyo, Japan), and the images were captured and analysed using microscope digital camera software (DP2-BSW; Olympus).

Confocal laser scanning microscopy (CLSM)

The three roots selected for CLSM analysis were cut longitudinally in half using a diamond saw under filtered water cooling. Specimens were immersed in a 50% ethanol solution with 100 μ mol L^{-1} sodium fluorescein (NaFl; Sinopharm Chemical Reagent Corp. Ltd., Shanghai, China) for 5 min and then washed in deionized water for 3–5 min. The middle third area of each specimen was observed using CLSM (LSM 5 PASCAL; Zeiss), and NaFl fluorescence was detected (NaFl: Ex 488 nm, Em 525/50 nm band pass filter).

Scanning electron microscopy (SEM)

The two roots selected for SEM analysis were fixed in 2.5% glutaraldehyde solution (Xin Fourth Ring Disinfection Technology Development Corp. Ltd, Beijing, China) at 4 °C for 12 h, then cut longitudinally in half using a diamond saw under filtered water cooling. The specimens were washed in phosphate-buffered saline (PBS) (Zhongshan Golden Bridge Biotechnology Corp. Ltd.) for 20 min, immersed in PBS for an additional 12 h and finally dehydrated in a graded ethanol series (50, 70, 80, 90, 95 and 100%; 20 min each). The specimens were then fixed on metallic stubs with double-faced conductive adhesive tape, sputter-coated with gold and vacuumized, and then observed using SEM (S-4800; Hitachi). The middle third area of each specimen was observed at ×4000 and ×8000 magnification, and surface images of cementum were captured by SEM.

Vickers microhardness indentation tests

Another 14 healthy premolars with single roots extracted for orthodontic purposes were selected for microhardness examination. The teeth were stored at room temperature in saline and examined under a dissecting microscope to eliminate teeth with cracks or hypoplastic defects. The crowns were removed at the CEJ using a diamond bur under filtered water cooling. The roots were embedded in polymerizing acrylic resin (Shanghai Medical Instruments Corp. Ltd, Shanghai, China) and sectioned in half horizontally in the midroot region using a saw (IsoMet Low Speed Saw; Buehler Ltd., Lake Bluff, IL, USA). The cementum cross section of each segment was ground polished with a water-cooled carborundum paper. The resulting 28 specimens (14 teeth split in two) were divided into two groups with each containing 7 coronal segments and 7 apical segments.

The surface hardness of the cementum was assessed with a Vickers Hardness Tester (HMV-2T Microhardness Tester; Shmadzu Corp. Ltd., Kyoto, Japan). Indentations were made with a Vickers diamond indenter at a minimum of three similar positions, using a loading force of 0.1 kg and a dwell time of 10 s. The three values were averaged to produce one hardness value for each specimen. The exposed cementum surfaces of the specimens were then treated with 20 mL NaOCl solution in a glass beaker at room temperature. After treatment, the samples were rinsed with saline and dried. Thereafter, the specimens were again submitted to Vickers microhardness indentation tests.

The data were analysed statistically using the Wilcoxon signed-rank test of two-related samples with

SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). Results were reported as mean values ± standard deviations. The testing was performed at the 95% confidence level ($P < 0.05$).

Results

Efficacy of NaOCl in removing necrotic PDL

In all specimens of the control group (group 1), necrotic PDL was observed throughout the root surface. In all transverse and longitudinal histological sections of group 2, after being wiped with gauze, most of the PDL was removed from the root surface, albeit some PDL remnants remained in each; the root surface was covered by a layer of thin fibrous tissue. Similarly, in all specimens of groups 3, 4 and 6, PDL fibre remnants were observed on the root surface. However, in all specimens of the remaining groups (groups 5 and 7–11), no PDL remnants were observed on the root surface (Fig. 1 and Fig. 2).

Effect of NaOCl on cementum surface structure

In all specimens of groups 5 and 9, the cementum remained intact without cracks or defects (Fig. 1 and Fig. 2). The mineralized fibres of cementum in these groups were arranged regularly and distributed evenly, and the cementum lacunae were clearly observed at ×4000 SEM magnification (Fig. 3). At ×8000 magnification, the structure of mineralized fibres and lacunae was better preserved in group 5 (Fig. 4).

Cracks and defects were observed in all specimens of groups 7, 8, 10 and 11 (Fig. 1 and Fig. 2), and disordered or destroyed cementum microstructure was observed at ×4000 SEM magnification (Fig. 3).

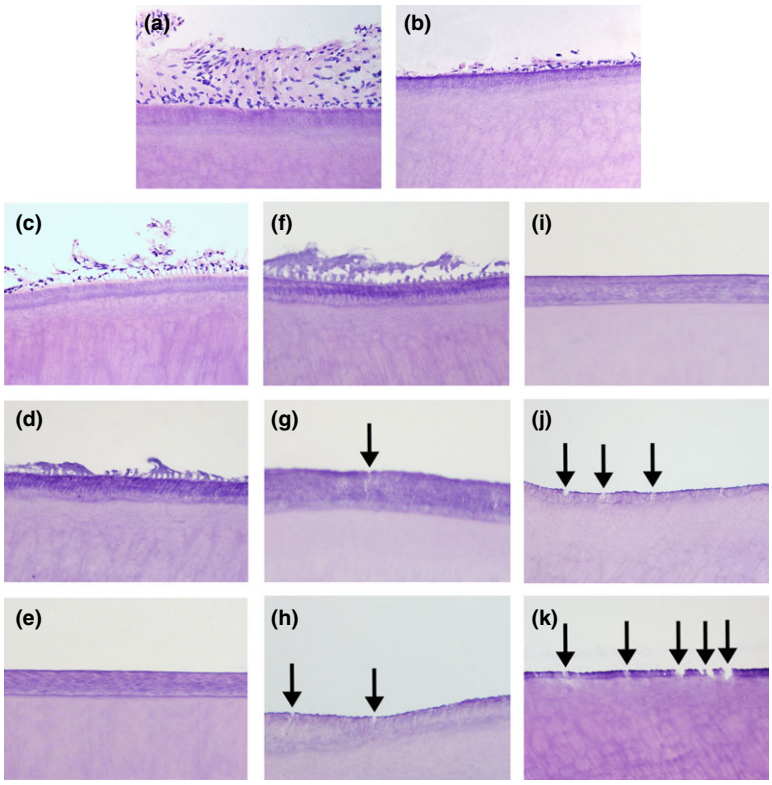

Figure 1 Histomorphometric analysis. HE (original magnification ×200). (a) In group 1 (control), PDL was observed throughout the root surface. (b) In group 2 (gauze wipe), fewer PDL remnants were observed in histological sections. (c, d, f) In groups 3, 4 and 6, PDL fibres could be observed on the root surface. (e, i) In groups 5 and 9, no PDL remnants were observed in histological sections, and the cementum remained intact without cracks or defects. (g, h, j, k) In groups 7, 8, 10 and 11, no PDL remnants were observed in histological sections, but cementum microcracks were observed (arrows); in group 11, defects of dentine were observed.

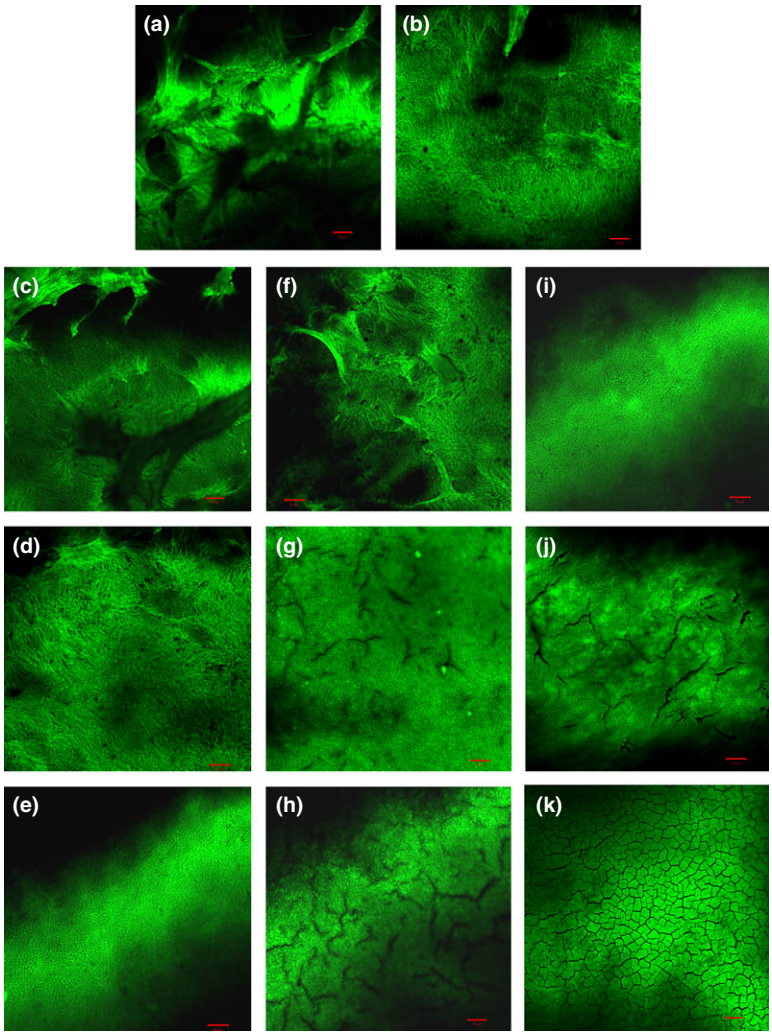

Figure 2 Confocal laser scanning microscopy analysis (red scale bar: 50 μm). (a) In group 1 (control group), PDL was observed throughout the root surface. (b) In group 2 (gauze wipe), fewer PDL remnants were observed. (c, d, f) In groups 3, 4 and 6, PDL fibres were observed on the root surface. (e, i) In groups 5 and 9, no PDL remnants were observed in histological sections, and the cementum remained intact without cracks or defects. (g, h, j, k) In groups 7, 8, 10 and 11, no PDL remnants were observed in histological sections, but cementum microcracks were observed.

Effect of NaOCl on cementum microhardness

The mean values and standard deviations of the cementum microhardness values before and after treatment with NaOCl solution are listed in Table 2. The initial microhardness values of corresponding surfaces between the two groups were not significantly different ($P > 0.05$). However, use of either 1% NaOCl for 15 min or 5.25% NaOCl for 5 min decreased cementum microhardness significantly ($P < 0.05$). The degree of change for each of the two groups from before to after treatment was not significantly different from each other ($P > 0.05$).

Discussion

Immediate replantation is well recognized as one of the most relevant factors that contributes to PDL healing of avulsed teeth (Andreasen *et al.* 1995b). However, clinical research has shown that most avulsed teeth are replanted after extended extra-alveolar times and were not maintained under physiological conditions (Zhang & Gong 2011). Delays in

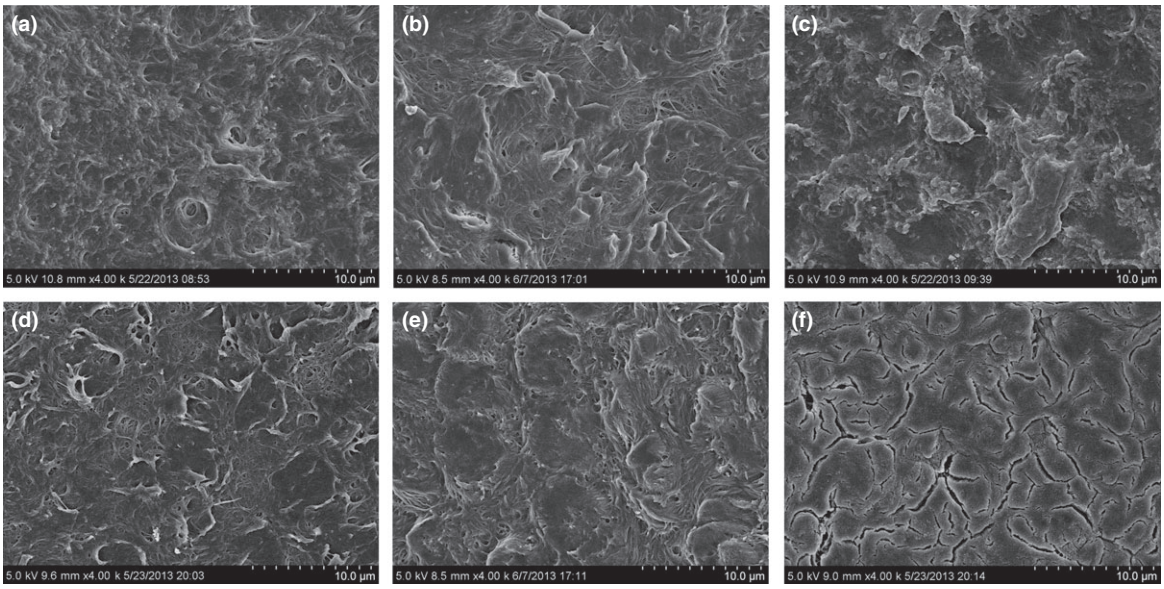

Figure 3 Scanning electron microscopy (original magnification ×4000). (a) In group 5, the mineralized fibres of cementum were arranged regularly and distributed evenly, and the cementum lacunae were clearly observed. (b) In group 7, the mineralized fibres of cementum were arranged irregularly and distributed unevenly, and the margin of cementum lacunae was unclear. (c) In group 8, the structure of cementum was destroyed. (d) In group 9, the mineralized fibres of cementum were arranged regularly and distributed evenly, and the cementum lacunae were clearly observed. (e) In group 10, the structure of cementum was disordered. (f) In group 11, many cracks were observed.

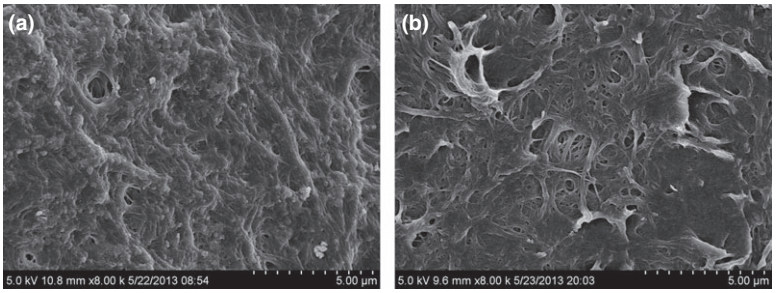

Figure 4 Scanning electron microscopy (original magnification ×8000). (a) In group 5, the structure of mineralized fibres and lacunae was better preserved. (b) In group 9, the marginal structure of lacunae was slightly damaged.

replantation of an avulsed tooth or use of an inappropriate storage medium will inevitably lead to PDL necrosis (Andreasen 1981, Blomlof 1981, Andreasen *et al.* 1995b). It is believed that the necrotic PDL should be removed in an attempt to increase the survival rate of replanted tooth, as the necrotic remnants may trigger external root resorption (Andreasen 1980, Andreasen *et al.* 1995b, Hupp *et al.* 1998, Pohl *et al.* 2005, Andersson *et al.* 2012). At the same time, the cementum layer should be protected as it is more resistant to inflammatory resorption than dentine (Lindskog *et al.* 1985, Gunraj 1999). Moreover, the integrity of cementum could prevent the spread of endodontic infection to periodontal tissue (Hammarstrom *et al.* 1986).

According to the most recent IADT Guidelines for management of avulsed permanent teeth (Andersson *et al.* 2012), amongst the multiple methods used for removing nonviable PDL (Nyman *et al.* 1985, Ripamonti & Petit 1989, Selvig *et al.* 1990, Zervas *et al.* 1991, Duggal *et al.* 1994, Kenny *et al.* 2000, Esper *et al.* 2007), the temporary treatment guidelines for

Table 2 Cementum microhardness values before and after NaOCl treatment

Treatment	N	Pre-treatment Roughness Values (Mean ± SD) HV0.1	Post-treatment Roughness Values (Mean ± SD) HV0.1	Δ
1% NaOCl 15 min	14	26.2 ± 5.4	22.5 ± 5.1[a]	3.7 ± 4.8
5.25% NaOCl 5 min	14	26.2 ± 7.9	23.1 ± 7.2[a]	3 ± 3.5

[a]indicates a significant difference ($P < 0.05$).
HV0.1: Vickers hardness test with a loading force of 0.1 kg and a dwell time of 10 s.

delayed replantation recommend removing attached nonviable soft tissue carefully with gauze. However, the findings of this study showed that, although the cementum layer was well preserved, obvious PDL remnants could be observed on the root surface, even by visual inspection. Therefore, a more effective method is needed to achieve a greater level of periodontal debridement.

NaOCl is the most commonly used endodontic irrigation solution because of its well-known antimicrobial effects, tissue-dissolving activity and low toxicity (Turkun & Cengiz 1997, Okino *et al.* 2004). The tissue-dissolving capability of NaOCl relies on its concentration, volume, temperature and the contact time with the tissue (Zehnder 2006, Stojicic *et al.* 2010).

Previous studies have shown that the tissue-dissolving ability of NaOCl solution decreases if it is diluted (Stojicic *et al.* 2010). Additionally, the antimicrobial and tissue-dissolving efficacies of NaOCl solution were enhanced by increasing the temperature of the solution and extending the working time (Sirtes *et al.* 2005, Christensen *et al.* 2008, Stojicic *et al.* 2010). However, changes in mechanical properties, such as decreased microhardness and increased roughness of radicular dentine, have been reported after exposure to NaOCl in concentrations of 2.5% and 5.25% (Ari *et al.* 2004), and the decreased elastic modulus and flexural strength of dentine was reported after exposure to 5.25% NaOCl (Sim *et al.* 2001). Also, NaOCl at 2.5% and 5.0% dissolved most of the pre-dentine and increased dentine ultrastructure disorders, as viewed by SEM (Koskinen *et al.* 1980). These effects of NaOCl on dentine structure and properties could be decreased, however, by controlling the exposure time to the NaOCl solution at a given concentration (Zehnder 2006).

Several animal studies have revealed that NaOCl at various concentrations could effectively remove necrotic PDL (Lindskog *et al.* 1985, Panzarini *et al.* 2005, Lustosa-Pereira *et al.* 2006, Sottovia *et al.* 2006); however, the optimal concentration and working time of NaOCl were not determined in these studies. The present results showed that, consistent with previous studies, the necrotic PDL-dissolving efficacy of NaOCl solution was enhanced by increasing its concentration and extending the working time, but these changes also increased damage to the root surface and cementum. After treatment with NaOCl above 2.5% for more than 10 min, cracks and defects in the cementum were observed using histological, CLSM and SEM analyses, and a more disordered cementum microstructure was also visible under SEM. Notably, CLSM micrographs revealed more cementum cracks than HE-stained sections. One possible reason for this might be that some of the cracks and fissures in the histological sections were filled by dye particles.

In the present study, after treatment with 1% NaOCl for 15 min or 5.25% NaOCl for 5 min, the cementum remained intact without cracks or defects under both histological and CLSM analysis (Fig. 1 and Fig. 2). However, when observed at ×8000 magnification by SEM, treatment with 1% NaOCl for 15 min appeared to preserve cementum structural integrity better than with 5.25% NaOCl for 5 min (Fig. 4).

A determination of microhardness provides indirect evidence of mineral loss or gain in dental hard tissues (Arends & Ten 1992). Based on the above morphological analysis results, the effect of NaOCl on cementum microhardness was compared. The Vickers microhardness test was selected for its suitability and practicality for evaluating surface changes of dental hard tissues after treatment with chemical agents (Lewinstein & Grajower 1981, Lewinstein *et al.* 1994, Kuramoto *et al.* 2001). The results showed that both treatment with 1% NaOCl for 15 min and 5.25% NaOCl for 5 min decreased cementum microhardness, suggesting that NaOCl may have some effect on the components of cementum structure. Similar effects on dentine were reported when NaOCl was used as an irrigation solution (Grigoratos *et al.* 2001, Sim *et al.* 2001, Slutzky-Goldberg *et al.* 2002, Ari *et al.* 2004). Despite these defects, NaOCl is still the most commonly used endodontic irrigation solution and may still be a

promising agent for root surface treatment of delayed replanted teeth.

Conclusions

Use of either a 1% NaOCl solution for 15 min or a 5.25% NaOCl solution for 5 min effectively removed necrotic PDL from delayed replanted teeth with minimal influence on the structural integrity of cementum. However, use of 1% NaOCl solution for 15 min was slightly more effective at preserving the cementum.

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