Disinfection Efficacy of Current Regenerative Endodontic Protocols in Simulated Necrotic Immature Permanent Teeth

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Abstract

Introduction: The lack of mechanical debridement and reduced concentrations suggested for chemical debridement to maintain stem cell viability call into question the disinfection efficacy of current regenerative protocols. Current protocols vary in the concentration and type of antibiotic medications used. The aim of this study was to determine if simulated immature teeth infected with Enterococcus faecalis can be completely disinfected by following current standardized regenerative protocols and to evaluate the probable effects of residual bacteria on stem cell toxicity. 

Methods: Sixty-eight caries-free maxillary incisors were used. S1 sampling protocols were validated in both negative and positive control groups via culture, scanning electron microscopy, and confocal laser scanning microscopy. All teeth, except the negative controls, were inoculated with E. faecalis. The teeth were divided into the following groups: group 1, triple antibiotic paste (ciprofloxacin:metronidazole:minocycline) at concentrations of 10, 1, and 0.1 mg/mL; group 2, double antibiotic paste (ciprofloxacin:metronidazole) at concentrations of 10, 1, and 0.1 mg/mL; group 3: Ultracal XS calcium hydroxide (Ultradent, St Louis, MO); and controls, negative and positive controls. Current regenerative protocols recommended by the American Association of Endodontists were followed. S2 sampling was performed after 4 weeks and tested for bacterial presence via culturing, scanning electron microscopy, and confocal laser scanning microscopic analysis. 

Results: The data showed that calcium hydroxide and the current recommended antibiotic concentrations are not capable of completely eliminating bacteria from simulated necrotic immature permanent teeth. 

Conclusions: Overall, this study focuses on the need to re-evaluate the balance between stem cell toxicity and bacterial elimination in order to determine the appropriate concentrations and medicaments for successful regenerative endodontic procedures. (J Endod 2016;42:1218–1225)
critical for successful revascularization because the new tissue will stop at the level it meets bacteria in the canal space (13). However, chemical debridement of the canal system alone must be relied on because more traditional mechanical debridement is insufficient to reliably create the conditions necessary for revascularization of the infected necrotic tooth (14). More recent studies have reported the use of the triple antibiotic paste (TAP) developed by Hoshino et al (15, 16) and its effectiveness in disinfection of the infected necrotic tooth, setting the conditions for subsequent revascularization. A preclinical study on dogs reported that the intracanal delivery of a 20-mg/mL solution of the TAP resulted in a greater than 99% reduction in mean colony-forming unit (CFU) levels, with approximately 75% of the root canal systems having no cultivable microorganisms present (17). Other studies have reported the use of calcium hydroxide (Ca(OH)$_2$) during these procedures (18).

The American Association of Endodontists (AAE) Regenerative Committee has established a standardized protocol for regenerative endodontic procedures. The protocol includes chemical debridement of the root canal system with 20 mL 1.5% NaOCl followed by placement of either double antibiotic paste (DAP), TAP, or Ca(OH)$_2$. However, a recent study by Ruparel et al (19) highlighted the toxicity that various concentrations of antibiotic paste have against human SCAPs. The results of the study supported using Ca(OH)$_2$ or a lower concentration of antibiotic paste (0.01–0.1 mg/mL) to maintain stem cell viability. Other studies have also shown TAP and DAP at higher concentrations can alter SCAP and periodontal ligament cell survival (20, 21). Based on these data, the current regenerative endodontic protocol uses TAP or DAP at a concentration of 0.1 mg/mL. However, it is still unclear whether these concentrations were effective in disinfecting the root canal system. Hence, this calls into question the disinfection efficacy of current regenerative protocols. Therefore, the aim of this study was 2-fold: to determine whether using Ca(OH)$_2$ or DAP or TAP (0.1, 1, and 10 mg/mL) is effective in disinfecting the canal space in simulated necrotic teeth and to determine the optimum concentration that is effective in disinfecting the canal and comparing this with known concentrations that are biocompatible for SCAPs.

### Materials and Methods

#### Culture of the SCAPs

Human SCAPs were obtained from Dr Shi’s laboratory (University of Pennsylvania, Philadelphia, PA). The cells were maintained in alpha-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum, 1% penicillin, and 1% L-glutamine (Invitrogen). Cells were incubated at 37°C in an atmosphere of 5% CO$_2$. Cells were used only until passage 4 because these cells show a more differentiated phenotype after passage 4 (22).

#### Culture of Natural Killer Cells

Natural killer cells were isolated by our collaborators as stated previously and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin, and 1% L-glutamine (Invitrogen) (23).

#### Chromium-51 Release Cytotoxic Assay

The chromium-51 (Cr-51) release assay was performed by our collaborators at the University of California, Los Angeles as described previously (24). Briefly, different numbers of purified natural killer cells (effector cells) were incubated with Cr-51-labeled target cells (SCAPs) at the different effector:target (E:T) ratios. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity in units of counts per minute (cpm) using a gamma counter. The percentage specific cytotoxicity was calculated and is shown in Figure 1:

$$\text{% Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

#### Preparation of the Teeth

Sixty-eight single-canal maxillary incisors were used in this study. Caries-free extracted central incisors were stored in diluted 0.5% NaOCl. Teeth were sectioned 3 mm above the cementoenamel junction, accessed, and prepared using K-files and ProTaper series rotary files (Dentsply International, York, PA) until an F5 reached patency. The smear layer was removed with 2 mL 6% NaOCl, 2 mL 17% EDTA, and 2 mL 6% NaOCl in each tooth under positive pressure irrigation. The teeth were placed in sterile saline until they were sterilized. Sterilization was performed using an autoclave at 240°F at 20 psi for 40 minutes with the teeth wrapped in moist 2 × 2 gauze (25). Teeth were then transferred to sterile vials containing 20 mL sterile brain-heart infusion (BHI) broth medium and kept in an incubator at 37°C for 48 hours to check the efficacy of the sterilization procedure.

#### Cultivation of Enterococcus faecalis and Specimen Inoculation

Pure isolated 24-hour cultures of Enterococcus faecalis (ATCC 29212) grown on BHI agar plates (Sigma-Aldrich, St Louis, MO) were suspended in 20 mL sterile BHI broth medium for 8 hours. Five drops of this bacteria medium were inoculated in a new 20-mL BHI broth for 4 hours. These bacteria suspensions were adjusted to match the turbidity of 1.5 × 10$^8$ colony-forming units (CFUs)/mL (equivalent to 0.5 McFarland standard). Twenty microliters of the bacterial in inoculum was added to each tooth to simulate a biofilm.

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### Figure 1

Increased natural killer cell cytotoxicity against the SCAPs. The Cr-51 cytotoxic assay results show that in the presence of innate immune cells SCAP cytotoxicity is increased. However, in the presence of activated innate immune cells, the cytotoxicity increases to levels as high as 90% with an E:T ratio of 10:1 and about 50% with the lowest E:T ratio of 1.25:1.
oculum was added to the vials containing the teeth suspended in sterile medium. The tubes were then closed and kept at 37°C for 30 days in aerobic conditions to ensure adequate penetration of bacteria into dental tubules (26, 27). Half of the inoculum broth for all specimens was replaced with 20 mL fresh sterile BHI medium every 5 days to avoid medium starvation. The purity of the cultures was confirmed by Gram staining after 30 days and later verified in the positive control sample under culture and scanning electron microscopic (SEM) and confocal laser scanning microscopic (CLSM) analyses.

Preparation for Bacteria Sampling

Teeth were rinsed with sterile saline, and the outside of each tooth was wiped with alcohol. A sterile cotton pellet was placed into the chamber, and the access cavity was sealed with Cavit (3M ESPE, St Paul, MN). The apical foramen was sealed with hot glue to resemble a closed system (28). Each tooth was disinfected with 30% hydrogen peroxide (H2O2) until no further bubbling of the solution occurred. All surfaces were coated with 10% tincture of iodine and allowed to dry. The tooth surfaces were then swabbed with a 5% sodium thiosulfate solution to inactivate the iodine tincture (29).

Initial Sample (S1)

The Cavit and cotton pellet were removed, and the canal was flushed with 2 mL sterile saline and dried with sterile paper points. With a sterile syringe, sterile BHI broth was inserted into the canals, removed with sterile paper points, and placed onto half of each BHI agar plate. Sterile BHI broth was reinserted and again removed with sterile paper points and placed in a 1.0-mL test tube of BHI broth. The 1-mL test tube was vortexed, and aliquots of 0.1-mL suspensions were then plated onto the remaining half of the BHI plate. The plates were then incubated aerobically for 48 hours at 37°C. This procedure was verified in both the negative and positive control groups.

Regenerative Protocol

Standard regenerative protocols as given by the AAE guidelines were followed by using copious gentle irrigation with 20 mL 1.5% NaOCl and 20 mL sterile saline with the EndoVac irrigation system (SybronEndo, Orange, CA) to the working length. The canals were dried with paper points, and DAP or TAP at concentrations of 10, 1, or 0.1 mg/mL or full-strength UltraCal XS Ca(OH)2 (Ultradent, St Louis, MO) was placed. The teeth were divided into the following groups according to the medicament they were receiving:

1. **Group 1**: 24 teeth with TAP 1:1 ciprofloxacin:metronidazole:micronycline at concentrations of 10, 1, and 0.1 mg/mL (8 teeth per concentration)
2. **Group 2**: 24 teeth with DAP 1:1 ciprofloxacin:metronidazole at concentrations of 10, 1, and 0.1 mg/mL (8 teeth per concentration)
3. **Group 3**: 8 teeth with UltraCal XS Ca(OH)2
4. **Group 4**: 6 teeth that were the negative controls (sterile broth incubation and rinsed with saline only [3 teeth] following the regenerative irrigation protocol without medicaments [3 teeth])
5. **Group 5**: 6 teeth that were the positive controls (6 teeth with inoculum for S1 sample and then rinsed with saline only [3 teeth] following the regenerative irrigation protocol without medicaments [3 teeth])

Access openings were closed with 4 mm Cavit and incubated aerobically at 37°C for 4 weeks as suggested by the AAE guidelines for regenerative endodontic procedures. After 4 weeks, the temporary fillings were removed, and the canals were gently irrigated with 20 mL 17% EDTA at the working length with the apical negative pressure irrigation system, rinsed with sterile saline, and dried with paper points.

**Final Sample (S2)**

After completion of the regenerative protocol, the following sampling procedure was used to test for bacterial presence. The root canal of each tooth was dried with sterile paper points and filled with reduced BHI broth. Three consecutive sterile coarse paper points were introduced into the canal to absorb the BHI broth. The paper points were transferred to a BHI agar plate. After collection of the first bacterial sample, the canal was refilled with BHI broth. A #15 K-file was placed into the canal to within 1 mm of the working length, and the canal was circumferentially filed for 10 seconds. Dentin shavings collected on the K-file were transferred to a test tube containing 1.0 mL BHI broth (30). The canal contents were then sampled with paper points as described previously for the S1 sample and placed into the test tubes containing the dentin chip shavings and BHI broth. The collected samples were vortexed for 10 seconds. Aliquots of 0.1-mL suspensions were then plated onto one half of the BHI agar plates and incubated aerobically at 37°C for 48 hours (26). CFUs were counted, and the purity of the cultures was confirmed by Gram staining and colony morphology. Half of the teeth from each group were sectioned and fixed for SEM analysis, and the other half from each group were prepared for CLSM analysis.

**SEM Examination**

Teeth were longitudinally grooved and split. Samples were rinsed twice in phosphate-buffered saline, fixed with 2.5% glutaraldehyde (Sigma-Aldrich) for 1 hour, and post fixed in 1% osmium tetraoxide (OsO4) (Sigma-Aldrich) for 30 minutes. Samples were then dehydrated, mounted on SEM discs, and sputter coated with 5 nm gold palladium for conductivity. The Sirion FEG microscope (FEI, Hillsboro, OR) operated at 10-kV acceleration voltage was used for analysis of the samples, which was focused on the middle and apical third of each section. Surface morphology of the specimens was examined at a magnification of 5000×. Images of 5-μm areas are taken, and representative images are shown in Figure 24–F.

**CLSM Examination**

Teeth were longitudinally grooved and split. The samples were stained with 30 μL Live/Dead dye (Life Technologies, Grand Island, NY) for 10 minutes. The samples were then examined with the LSM510 confocal microscope (Carl Zeiss, Thornwood, NY) for 10 minutes. The thresholds of the red and green fluorescence were manually adjusted. Images of the apical and middle thirds (4 slices each) were taken. Five random areas of 0.2 × 0.2 mm starting from the border of the root canal were selected for CLSM examination. Bacteria with intact membranes (live bacteria) were stained green by SYTO 9 (Life Technologies), and bacteria with damaged membranes (dead bacteria) were stained red by propidium iodide uptake (31). Representative images from 8 sections (4 teeth) of the experimental groups are shown in Figure 34–F.

**Data Analysis**

The required sample size was calculated to be 8 samples per group. This gave the study at least 95% power to detect a difference between group means using analysis of variance with a significance level of 0.05. The power was set to 95% to allow for possible loss of samples. With a loss of 1 sample per group, a sample size per group of 7 would...
yield at least 90% power, and a loss of 2 samples per group would still yield at least 85% power. The differences in bacterial colonies were assessed at S1 and S2. Viable counts/positive cultures were transformed to their log₁₀ values. The Wilcoxon signed rank test was used for intra-group analysis comparing the reduction in the number of CFU counts from S1 to S2. The Kruskal-Wallis test was used to test for differences in CFUs across groups. The significance level was set at \( P < .05 \) for all tests.

**Figure 2.** Representative images of the radicular dentin using SEM imaging. The radicular dentin samples were washed and fixed (as stated in the Materials and Methods section), and then their surface topography was analyzed using scanning electron microscopy. (A) Positive control, (B) negative control, (C) TAP 10 mg/mL, (D) TAP 1 mg/mL, (E) TAP 0.1 mg/mL, (F) DAP 10 mg/mL, (G) DAP 1 mg/mL, (H) DAP 0.1 mg/mL, and (I) UltraCal. Scale bars = 5 \( \mu \)m and 5000× magnification.

**Figure 3.** Representative images of the radicular dentin using CLSM examination. Confocal laser scanning microscopy of the radicular dentin and infected dentinal tubules after exposure to the different disinfecting agents and viability staining. Bacteria with intact membranes (live bacteria) were stained green by SYTO 9, and bacteria with damaged membranes (dead bacteria) were stained red by propidium iodide uptake. (A) Positive control, (B) TAP 10 mg/mL, (C) TAP 0.1 mg/mL, (D) DAP 10 mg/mL, (E) DAP 0.1 mg/mL, and (F) UltraCal. (B) The TAP 10 mg/mL group showed the least amount of viable bacteria compared with the other groups.
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Results

Immune Cell–mediated Cytotoxicity

Immune cell–mediated cytotoxicity was analyzed using the Cr-51 assay. Inactivated innate immune cells in direct contact with SCAPs caused cytotoxicity of up to 45%. However, when the immune cells were activated with interleukin 2 in the Cr-51 assay, the cytotoxicity to SCAPs was about 90% with an E:T ratio of 10:1 and was still at about 50% at the lowest E:T ratio of 1.25:1 (Fig. 1).

TAP 10 mg/mL Showed a Significant Reduction in the S2 Sample

All samples except negative controls showed bacterial growth on BHI plates at the S1 sample. The mean log10 viable counts at the S1 sample showed no statistical differences between the groups before starting the regenerative protocol (data not shown). After completion of the regenerative protocol, TAP 10 mg/mL and DAP 10 mg/mL showed the most significant overall reduction in bacterial counts ($P < .001$). However, some of the DAP 10 mg/mL samples showed positive S2 cultures (Table 1). The UltraCal group showed the least significant reduction in bacterial counts among the experimental groups and was comparable with the positive irrigation control group (Table 1). The negative control did not show any bacterial growth throughout the experiment.

SEM and CLSM Analyses Confirmed the Microbiological Results

SEM and CLSM examinations showed findings consistent with the S2 cultures, with overall numbers of remaining bacteria in the dentinal tubules (Figs. 2 and 3). Some residual viable bacteria remained in the dentinal tubules of the TAP 10 mg/mL group when stained with the Live/Dead stain (Fig. 3B) despite showing negative cultures at S2 (Table 1) and no bacteria in the SEM sections (Fig. 2C). The DAP 10 mg/mL sample did not show a negative S2 culture, but no visible bacteria could be examined in the SEM sections (Fig. 2F). However, this group did show a larger number of viable bacteria in the dentinal tubules as seen in Figure 3D. The lower concentrations of TAP and DAP did show viable bacteria staining as examined by CLSM analysis. The Ca(OH)2 group showed the presence of bacteria across all the different analyses conducted. The negative controls did not show any staining (data not shown).

Staining of the Tooth Could Occur with Lower Concentrations of TAP

Previous studies have shown that TAP can cause significant staining of the tooth (20). Our data are consistent with these results. The traditional thick pastelike consistency of TAP placed over a 4-week period shows significant crown and root staining (Fig. 4A–C). However, when a concentration of 10 mg/mL, a liquidlike consistency, was used, it considerably reduced crown and root staining over a 4-week period (Fig. 4D–F).

Discussion

Previous research has shown that elimination of bacteria from the root canal system is important for both pulpal and periapical healing (16). However, because of the lack of mechanical debridement in immature teeth during regenerative endodontic procedures, chemical debridement must be solely relied on. This differs from conventional root canal therapy in which the canals are chemomechanically debrided. This could explain the high success rates with conventional treatment (32, 33). Recent research has shown the stem cell toxicity of commonly used medicaments like TAP and DAP in regenerative endodontics. Concentrations above 1 mg/mL were associated with significantly increased SCAP toxicity in vitro (19). Another drawback of using TAP is tooth staining. Lower concentrations of TAP appear to stain teeth less than methods using thick pastelike consistencies (20). Taking all these factors into consideration, the current regenerative protocol suggests the use of either TAP or DAP at a concentration of 0.1 mg/mL. On the other hand, Ca(OH)2, which is the most commonly used intracanal medicament in endodontics, was shown to actually increase SCAP proliferation when used during regenerative procedures (19). However, our data show that Ca(OH)2 or the 0.1-mg/mL concentrations of either TAP or DAP did not disinfect the canal effectively (Table 1). Even though it is important to consider stem cell toxicity caused by these intracanal medications, one must also consider the indirect cytotoxicity caused by inadequate disinfection of the root canal system. Research has shown that bacterial DNA, lipopolysaccharides (LPS) and lipoteichoic acid (LTA) can activate the innate immune system (34, 35). In addition, previous research has shown that activated immune cells could be potent inducers of cell death in dental pulp stem cells and mesenchymal stem cells (36). Our data show similar results with SCAPs. The Cr-51 release assay showed that the activated innate immune system can lead to the death of about 90% of SCAPs at an E:T ratio of 10:1 (Fig. 1). This same mechanism could likely occur during regenerative endodontic procedures when the canal is inadequately disinfected and bleeding is induced, thereby leading to activation of the immune system because of the remaining viable bacteria. This activated immune system could potentially cause death of the SCAPs, which are introduced into the canal when bleeding is induced, and this, in turn, could cause failure of the regenerative endodontic procedure. The percentage of cell death of SCAPs could possibly increase to 100% depending on the number of immune cells that are introduced into the root canal system, which is very likely higher than a 10:1 ratio. This could be what actually happens during these procedures. Hence, it

<table>
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<th>Medicaments</th>
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<th>Mean (SD) S1 samples</th>
<th>Mean (SD) S2 samples</th>
<th>$P$ value</th>
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<tr>
<td>TAP</td>
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<td>2.48 (0.76)</td>
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<td>0.85 (0.99)</td>
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<td></td>
<td>1 mg/mL</td>
<td>2.06 (0.6)</td>
<td>1.24 (0.94)</td>
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<td></td>
<td>0.1 mg/mL</td>
<td>2.39 (0.8)</td>
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<td>.089</td>
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<td>UltraCal Ca(OH)$_2$</td>
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<td>2.21 (0.49)</td>
<td>1.28 (1.1)</td>
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<td>0.9 (0.37)</td>
<td>.313</td>
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<td>Irrigation control</td>
<td>2.05 (0.26)</td>
<td>1.66 (0.06)</td>
<td>1.35 (0.1)</td>
<td>.155</td>
</tr>
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</table>

DAP, double antibiotic paste; SD, standard deviation; TAP, triple antibiotic paste.

*Statistical significance ($P < .001$).
is important to maintain a balance among stem cell toxicity, medicament concentration, and adequate disinfection.

The teeth were infected with *E. faecalis* because it was the bacteria of choice in similar *in vitro* studies (37, 38). The S1 sample did not show a statistical difference between the groups, suggesting that all the samples were adequately inoculated with the bacteria (Table 1). Four weeks after the treatments were completed for each group as stated in the Materials and Methods section, the S2 samples were analyzed. The S2 samples showed a statistically significant difference between the groups (Table 1). The data were further analyzed and showed that TAP 10 mg/mL performed significantly better than the other medicaments when considering bacterial reduction (Table 1). All the S2 samples for TAP 10 mg/mL were negative for cultures. The DAP 10-mg/mL sample also showed a significant reduction from the S1 to the S2 sample. However, the DAP group still had some cultivable bacteria (Table 1). All the other groups showed a slight decrease in bacterial counts, which was not statistically significant. The positive control and the irrigation control groups did not show statistically significant differences between the S1 and S2 samples (Table 1). The negative controls did not show any growth throughout the experiment (data not shown). Ruparel et al (19) showed that at a concentration of 10 mg/mL TAP, approximately 10% of SCAPs survived. Hence, we believe that this concentration may be beneficial because there may be a small percentage of the SCAPs that survive versus having no SCAPs, which would possibly lead to failure of the regenerative procedure. Another important consideration is the complete removal of the antibiotic from the root canal system because any residual antibiotic could cause decreased levels of SCAP survival. However, further research is needed in this area.

It is well-known from previous research studies that bacteria can respond to environmental stresses by activating survival mechanisms and adaptive responses, namely starvation and the viable but nonculturable state (39). Hence, traditional culture techniques may not be sufficient to determine whether the TAP at 10 mg/mL would satisfactorily disinfect the canal. Previous studies have described the 2 stain methods to be adequate in discerning between live and dead bacteria (40). The 2 stains differ in their ability to penetrate normal and damaged bacterial cells. Live bacteria with intact membranes stain fluorescent green (SYTO9), whereas dead bacteria stain fluorescent red because their membrane is damaged, allowing penetration of the propidium iodine stain, which is responsible for the red fluorescence. The teeth were visualized under the confocal laser scanning microscope. The positive control (Fig. 3A) showed bright green fluorescent staining, whereas the negative control did not stain for either dye (data not shown). TAP 10 mg/mL (Fig. 3B) showed some green and red staining, suggesting that there may be some bacteria in these teeth that are in the viable but nonculturable state. The results for all the other samples including the Ca(OH)2 sample correlate well with the microbiology culture results. The sections of the tooth were analyzed with the scanning election microscope. SEM analysis is a method that has been used frequently for microbiological studies. The data from the SEM study were similar to those obtained from the culture and CLSM studies. The negative control (Fig. 2B), TAP 10 mg/mL (Fig. 2C), and DAP 10 mg/mL (Fig. 4F) did not show any bacteria. One possible reason that DAP 10 mg/mL did not show any bacteria could be because of the way the teeth were sectioned. This is one of the limitations of microscopy in that the sampling volume is limited. However, because previous research has shown that CLSM analysis complements the data obtained using SEM analysis, both procedures were performed (41).
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The authors deny any conflicts of interest related to this study.

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