Antimicrobial Effect of Peptide DJK-5 Used Alone or Mixed with EDTA on Mono- and Multispecies Biofilms in Dentin Canals

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Abstract

Introduction: The present study aimed to evaluate the antibacterial effect of a new peptide, DJK-5, used alone or mixed together with EDTA on mono- and multispecies biofilms in dentin canals covered by a smear layer with or without preceding sodium hypochlorite (NaOCI) irrigation. Methods: One hundred twelve dentin blocks (224 final specimens) were prepared and divided into 56 groups, and Enterococcus faecalis or multispecies bacteria were introduced into dentinal tubules by centrifugation. After 1 week of cultivation, a uniform smear layer was created on the surface of the dentin blocks, and the samples were exposed to sterile water, 17% EDTA, 2% or 6% NaOCl, 10 μ g/mL DJK-5, or a mixture of 8.5% EDTA +10 μ g/mL DJK-5 or were combined treated with the solution in the following sequence: 2% or 6% NaOCI +10 μ g/mL DJK-5, 2% or 6% NaOCI + 8.5% EDTA +10 μg/mL DJK-5, 2% or 6% NaOCI + 8.5% EDTA + 10 μ g/mL DJK-5. Specimens without a smear layer treated by 6% NaOCl or 10 μ g/ mL DJK-5 served as the positive control. The irrigant exposure time was 3 or 10 minutes. The antibacterial efficacy was determined by live/dead staining and confocal laser scanning microscopy. Results: The smear layer reduced the antibacterial capacity of 6% NaOCI and 10 μ g/mL DJK-5. The efficacy of 2% or 6% NaOCl followed by 10 μ g/mL DJK-5 was superior to 10 μ g/ mL DJK-5 alone (P < .05) but inferior to 2% or 6% NaOCI + 8.5% EDTA + 10 μ g/mL DJK-5 and 2% or 6% NaOCI + 8.5% EDTA + 10 μ g/mL DJK-5 (P < .05). The mixture of 8.5% EDTA and 10 μ g/mL DJK-5 had the same disinfection effectiveness as 10 μ g/mL DJK-5 used alone (P < .05). Using 2% or 6% NaOCI before EDTA + peptide always resulted in the highest killing (P < .05). Conclusions: The smear layer inhibits the disinfectant effect in dentin. Peptide DJK-5 showed a

strong antibacterial effect against mono- and multispecies biofilms in dentin canals. The highest killing was measured when 6% NaOCI was followed by a mixture of EDTA and peptide DJK-5. (J Endod 2018; \blacksquare :1–5)

Key Words

Antimicrobial, biofilm, confocal laser scanning microscopy, dentin, DJK-5, EDTA, smear layer

Bacteria are the causaperiodontitis (1). They colonize the necrotic root canal and dentinal tubules as biofilms (2, 3). Accordingly, the goal of root canal treatment is to remove biofilms and kill microbes by

Significance

An antibiofilm peptide is successfully combined with EDTA without losing any of its effectiveness against dentin biofilm. The peptide DJK-5 mixed with EDTA or used separately enhances the antimicrobial activity of the irrigant irrigation and could therefore facilitate killing of microbes in dentin biofilm also *in vivo*.

instrumentation, irrigation, use of locally intracanal medicaments, and antibacterial root filling materials. Instrumentation removes some of the biofilm structures, and irrigation with chemically active agents is supposed to complete the removal and killing of microbes (4).

For decades, a focus has been on how to improve the efficiency of instrumentation in debridement of the root canal system and how to amplify the antimicrobial activity of the disinfection solutions. Although instrumentation is necessary to allow effective irrigation, it also has potentially negative effects such as the production of dentin debris and the smear layer (5, 6). The smear layer may contain bacteria and antigenic material from microorganisms, and it can act as a substrate to which new microbes can attach (7). In addition, the smear layer acts as a physical barrier to disinfecting agents, making it more difficult for them to kill bacteria in infected dentinal tubules (8, 9).

Sodium hypochlorite (NaOCl) is the most commonly used solution in endodontics, partly because of its activity against endodontic bacteria. However, the presence of the smear layer weakens the antibacterial activity of NaOCl in dentin (9, 10). NaOCl dissolves organic tissue effectively (11), but it has no effect on the inorganic portion of the smear layer (12). A decalcifying agent, such as EDTA, is needed for

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chelation of the inorganic components and to complete the removal of the smear layer from the root canal surface (13).

EDTA lacks antimicrobial activity (14); therefore, many dentists use NaOCl again as a final rinse after EDTA (15). However, when used after EDTA, NaOCl has been shown to cause dentin erosion, which may negatively impact the structural strength of the root (16, 17). Therefore, a few different combination products have been introduced in which substances with antimicrobial activity have been added to either EDTA or citric acid (18, 19).

DJK-5, a cationic peptide, was recently reported as having strong antimicrobial and antibiofilm activity (20, 21). The antimicrobial activity of DJK-5 is partly related to its cationic amphipathic properties; it inhibits accumulation and accelerates degradation of guanosine tetraphosphate inside bacterial cells (22), which is important for biofilm development (20) and survival in low-nutrient environments (23). DJK-5 has strong antibacterial activity against oral multispecies and *Enterococcus faecalis* biofilms (24). Therefore, it is a potentially promising agent in an endodontic irrigant.

In our previous study, the mixture of EDTA and DJK-5 proved effective in killing bacteria in oral multispecies and *E. faecalis* biofilms cultured on collagen-coated hydroxyapatite disks *in vitro* (25). In the present study, corresponding mono- and multispecies biofilms were grown in dentin canals, which in some specimens were covered by the smear layer. The goal of the study was to examine the antibiofilm effect of 2% and 6% NaOCl, 17% EDTA, and antimicrobial peptide DJK-5 (10 μ g/mL) used either alone, in sequence, or in a mixture (EDTA and DJK-5) against multispecies and *E. faecalis* biofilms in dentin canals with or without the smear layer. The null hypothesis was that irrigation with 2% or 6% NaOCl followed by a mixture of EDTA and DJK-5 is not more effective against bacteria in dentin canal biofilms than conventional irrigation with NaOCl and EDTA.

Materials and Methods

Dentin Block Preparation

Sixty caries-free single-rooted human teeth extracted for orthodontic reasons were collected according to the protocol approved by the University of British Columbia Clinical Research Ethics Committee review boards (certificate H12-02430). One hundred twelve dentin blocks were prepared as previously described (26), providing 224 dentin specimens.

Disinfecting Solutions

DJK-5 peptide was synthesized and purified as previously described (20). The stock solution (100 μ g/mL) was prepared by suspending the powder in sterilized deionized water. The ready-to-use EDTA-peptide mixture was freshly prepared by adding 17% EDTA (pH = 7.0) to 20 μ g/mL DJK-5 at the proportion of 1:1 to the final concentration containing 8.5% EDTA and 10 μ g/mL DJK-5. Six percent NaOCl (Clorox Bleach; Clorox, Oakland, CA) was obtained from the manufacturer. The available chlorine concentration was verified by iodometric titration as previously described (27). The 2% NaOCl was prepared by diluting 6% NaOCl in sterilized deionized water.

Dentin Block Infection

E. faecalis VP3-181, originally isolated from an infected root canal (28), was subcultured on brain-heart infusion (BHI) agar (Becton-Dickinson, Sparks, MD) plates aerobically at 37°C overnight. Pooled supragingival and subragingival dental plaque was collected from 1 healthy adult volunteer, and written informed consent was obtained for collecting the plaque samples. This study was approved by the University of British Columbia Clinical Research Ethics Committee Review Board, Vancouver, BC, Canada (certificate H12-02430). *E. faecalis*

and plaque multispecies bacteria were suspended in BHI broth (Becton-Dickinson), standardized in density, and centrifuged into the dentinal tubules following a previously published protocol (26). Dentin blocks with *E. faecalis* were incubated in BHI broth placed in an incubator (VWR General Purpose Digital Laboratory Incubators, Radnor, PA) (37°C) in air for 1 week, whereas blocks with the mixture of oral bacteria were incubated anaerobically (Bactron300 Anaerobic Chamber; Sheldon Manufacturing Inc, Cornelius, OR) at 37°C for 1 week. No fresh BHI broth was added during the 7-day incubation period.

Smear Layer Production and Dentin Disinfection

At the end of the incubation, dentin blocks were removed from the platform in the tubes, rinsed in 0.85% saline for 1 minute, and dried with paper points. The outer side of the specimens was sealed with a thin layer of nail vanish to simulate the cement layer on the root surface. Before exposure to the various solutions, the smear layer was created on the canal side of dentin blocks using a medium-grit cylinder flat-end bur (Patterson Dental, Halifax, Canada) at 1500 rpm for 4 seconds each. Infected dentin blocks (for *E. faecalis* or multispecies biofilms) were divided into 2×28 groups (4 dentin pieces in each of the 56 groups) for the short and long exposures to various disinfecting solutions as listed in Table 1.

The specimens were placed in a 96-well culture plate with the canal side up, and 100 μ L of each solution was added onto the surface of dentin specimens (Table 1). After the exposure to each solution in the sequence, the disinfectant was aspirated with a pipette, and the specimen was immersed in 0.85% saline water for a few seconds and dried with paper points before adding the next solution.

Confocal Laser Scanning Microscopic Examination

After exposure to the solutions, the dentin pieces were split from the cemental side along the root canal axis into 2 halves in order to expose a fresh surface of longitudinally fractured dentin tubules as previously described (26). The specimen was then stained with SYTO-9 and propidium iodide (BacLight LIVE/DEAD Bacterial Viability Kit; Molecular Probes, Eugene, OR) and scanned using a confocal laser scanning microscope (FV10i-LIV; Olympus, Tokyo, Japan) as described previously (24). For each group, a minimum of 4 dentin pieces were examined with a minimum of 10 scanned stacks using stratified sampling.

Statistical Analysis

The proportions of dead bacteria were measured and statistically compared between different irrigation protocols within each of the 4 main groups as detailed in Table 1 using 1-way analysis of variance with SPSS 16.0 software (SPSS Inc, Chicago, IL) followed by the post hoc Fisher least significant difference multiple comparison test at a significance level of P < .05.

Results

DJK-5 alone, in a positive control without the smear layer, killed most of the bacteria in dentin biofilms, 78.3% in *E. faecalis* and 75% in multispecies biofilms in 3 minutes (Fig. 1A and B). In another positive control with no smear layer, 6% NaOCl in 3 minutes killed 55.2% and 51.2% of the bacteria in the corresponding biofilms. EDTA was not tested against dentin bacteria in the absence of the smear layer.

When the smear layer was present, the highest killing in all 4 categories (*E. faecalis* and multispecies biofilms and short and long exposure) was obtained when 6% NaOCl was used first followed by EDTA and DJK-5; the latter 2 were used either as a mixture or in the following sequence: EDTA and DJK-5 (Fig. 1A-D). When 6% NaOCl was replaced by 2% NaOCl in the same irrigation sequence, killing was still strong but

TABLE 1. Times of Exposure to the Indicated Solutions Separately, in a Sequence, and in Combinations

Solutions	Short exposure (min)	Long exposure (min)
Sterile water	3	10
17% EDTA	3	10
2% NaOCI	3	10
6% NaOCI	3	10
6% NaOCI (no smear layer)	3	—
10 μg/mL DJK-5	3	10
10 μ g/mL DJK-5 (no smear layer)	3	—
8.5% EDTA + 10 μg/mL DJK-5	2	4
2% NaOCI +10 μ g/mL DJK-5	2 + 1	8 + 2
2% NaOCl + 8.5% EDTA +10 μg/mL DJK-5	1 + 1+1	6 + 2 + 2
2% NaOCl + 8.5% EDTA +10 μg/mL DJK-5	1 + 2	6 + 4
6% NaOCI + 8.5% EDTA	2 + 1	8 + 2
6% NaOCl + 10 μg/mL DJK-5	2 + 1	8 + 2
6% NaOCl + 8.5% EDTA +10 μg/mL DJK-5	1 + 1+1	6 + 2 + 2
6% NaOCl + 8.5% EDTA +10 μg/mL DJK-5	1 + 2	6 + 4

NaOCl, sodium hypochlorite.

Short exposure was always 3 minutes total, and long exposure was always 10 minutes, except for the mixture of EDTA and peptide DJK-5, which was 4 minutes.

weaker (P < .05); 6% NaOCl followed by DJK-5 without EDTA was equally effective as 2% NaOCl + EDTA + DJK-5 (Fig. 1*A*–*D*). Killing by DJK-5 alone was equally effective as with the conventional 6%

NaOCl + EDTA irrigation in specimens with the smear layer. The weakest effect was measured with 6% NaOCl, 2% NaOCl, EDTA, and water in the descending order when each of them was used alone in samples with



Figure 1. The volume proportion of dead bacteria in dentinal tubules after the short and long exposure time. (*A*) *E. faecalis* biofilms with short exposure, (*B*) multispecies biofilms with short exposure, (*C*) *E. faecalis* biofilms with long exposure, and (*D*) multispecies biofilms with long exposure. Differences between the 14 irrigation protocols within each of the 4 main groups (A–D) were separately tested with 1-way analysis of variance and the post hoc Fisher least significant difference multiple comparison test. Irrigation sequences identified with the same lowercase letter are not statistically significant (P > .05). No comparisons were made between the 4 main groups.

the smear layer (Fig. 1A-D). Long exposure to the same irrigant sequences as in the shorter exposure resulted in higher killing of bacteria in the biofilms, but the differences were relatively modest (Figs. 1 and 2A1-D2)

Discussion

DJK-5 is a recently developed D-enantiomeric peptide with a strong activity in inhibiting biofilm formation and killing bacteria in previously formed biofilms. DJK-5 is able to penetrate the cell membrane, and it causes its effects by targeting and degrading the intracellular nucleotides of guanosine tetraphosphate, which plays an important role in the formation and maintenance of bacterial biofilm.

The results of the present study confirmed the long-held belief and the results of some earlier studies of the impact of the smear layer on disinfectant effectiveness inside dentin (5-7, 9). NaOCl and peptide DJK-5, when used alone, both had a clearly reduced effect on bacteria in dentin when the smear layer was present (Figs. 1 and 2). In control specimens with no smear layer, DJK-5 killed almost 80% of both E. fae*calis* and mixed biofilm bacteria in just 3 minutes (Fig. 1), which is the highest killing reported inside dentin in the present and previous studies (9, 24, 25). Therefore, we wanted to examine if using peptide DJK-5 after EDTA or mixed with EDTA would increase the effectiveness by the conventional sequential use of NaOCl and EDTA against biofilm bacteria in dentin canals covered by the smear layer. Recently, a study using an open biofilm model on collagen-coated hydroxyapatite discs showed rapid and strong killing by the DJK-5 peptide (25). The same study also examined the use of the peptide alone and mixed with EDTA and found no inhibition between the 2; biofilm killing remained at the same high level, and in an experiment to remove the smear layer, the peptide did not weaken the ability of EDTA, which was used after NaOCl (25). In the present study, EDTA and DJK-5 together also had the same antibacterial effect against dentin biofilms than DJK-5 alone (Figs. 1 and 2). However, because EDTA does not remove the whole

smear layer, the effect of the mixture was less than for DJK-5 alone on specimens without the smear layer (Fig. 1).

One of the main targets for the action of NaOCl is the amino groups in proteins and peptides (11, 29). Therefore, a mixture of NaOCl and DJK-5 was not used in the present study. Instead, DJK-5 was used after NaOCl, with a short water rinse in between. Killing of dentin bacteria was much higher when NaOCl and DJK-5 were used sequentially than when either one was used as the only agent. The result suggests that the possible residual NaOCl on the specimen surface and inside dentin did not have a noticeable effect on DJK-5 activity. Interestingly, although NaOCl only affects the organic component of the smear layer (30) and scanning electron microscopic studies have shown the smear layer to be seemingly intact after NaOCl (25), NaOCl and NaOCl followed by DJK-5 had a strong antibacterial effect against bacteria in dentin biofilm (Fig. 2A2 and B2). After only 3 minutes (2 minutes with 6% NaOCl + 1 minute with DJK-5), 50% of both E. faecalis and mixed biofilm bacteria had been killed, and after 10 minutes of exposure (8 + 2 minutes) the proportion of dead bacteria had risen to approximately 60%. Again, compared with results from earlier studies (9, 24, 25), albeit limited, these are high numbers. The results suggest that both 6% NaOCl and the peptide DJK-5 can penetrate through the smear layer and exert their antibacterial effect in dentin effectively although the killing was not quite as high as when there was no smear layer.

The best killing effectiveness in smear layer specimens was obtained when 6% NaOCl, EDTA, and DJK-5 were all used. After 3 minutes of exposure (1 + 1 + 1 minute), approximately 60% of the bacteria were killed in both *E. faecalis* and mixed biofilms, and after the longer exposure of 10 minutes (6 + 2 + 2 or 8 + 2 minutes), the treatment had killed approximately 70%–75% of the bacteria in both biofilm groups. The obvious explanation is that for optimal smear layer removal, both NaOCl and EDTA are required. After this, the DJK-5 peptide has better access to penetrate into dentin and kill additional microbes. The killing was slightly less than in the control specimens without the smear layer exposed only to DJK-5 (Fig. 1), but this may be because of the fact that



Figure 2. (*A1* and *A2*) *E. faecalis* biofilms with short exposure: (*A1*) 10 μ g/mL DJK-5 (no smear layer) and (*A2*) 6% NaOCl. (*B1* and *B2*) Multispecies biofilms with short exposure: (*B1*) 10 μ g/mL DJK-5 (no smear layer) and (*B2*) 6% NaOCl. (*C1* and *C2*) *E. faecalis* biofilms with long exposure: (*C1*) 6% NaOCl + 8.5% EDTA + 10 μ g/mL DJK-5 and (*C2*) 6% NaOCl + 8.5% EDTA. (*D1* and *D2*) Multispecies biofilms with long exposure: (*D1*) 6% NaOCl + 8.5% EDTA + 10 μ g/mL DJK-5 and (*D2*) 6% NaOCl + 8.5% EDTA.

the peptide exposure was 3 minutes in the DJK-5–only experiments and 2 minutes in the sequential use of the 3 irrigants.

The exposure times, 3 and 10 minutes, were chosen based on earlier studies (9) and what can be regarded as clinically realistic. The longer exposure, 10 minutes, might seem quite long in a clinical situation; however, it is worth noting that 6 or 8 minutes of this was NaOCl irrigation, which can easily be the case in multirooted teeth when the dentist might have to use a long time looking for missing canals or negotiating calcified, curved canals. Therefore, it was important to include the groups with 10-minute irrigant exposures, most of which were with NaOCl in the present study.

Dentin disinfection has for decades been an area of interest in endodontics (3, 4). The importance of microbes in dentinal tubules for short-term healing, long-term prognosis, and even reinfection has been debated and is likely to vary in different cases. However, dentin disinfection is a useful indicator of the effectiveness of different antimicrobial strategies in clinical endodontics. Many different models have been used to quantitate the killing of dentin microbes (26, 31, 32). In the last few years, several studies have been published in which viability staining and confocal microscopy have been used (9, 26, 33, 34). In our model, including the present study, both *E. faecalis* and a mixture of different oral bacteria from interdental biofilm were introduced into the dentinal tubules by serial centrifugation. After this, the dentin pieces with the bacteria were incubated in BHI to allow for the biofilm to develop. Negative control experiments with water as the irrigant in the present and earlier studies (9, 26)indicate that the microbes survive and tolerate the physical forces of centrifugation. The reason for the use of centrifugation is that it secures even a strong presence of bacteria in the dentinal tubules in all specimens, making comparisons between samples and hopefully also between different studies possible (26). It is important to note that the proportions of dead bacteria after 6% NaOCl or EDTA in the present study and in the study by Wang et al (9) 4 years ago using the same model and smear layer are practically identical. This indicates high reliability and repeatability of the model. The effectiveness of irrigations including the new peptide DJK-5 was higher than that of other compounds or their combinations in the present and previous studies (9, 33). Therefore, the null hypothesis was rejected.

In conclusion, the smear layer inhibits the disinfectant effect in dentin. Peptide DJK-5 showed a strong antibacterial effect against mono- and multispecies biofilms in dentin canals. The highest killing was measured when 6% NaOCl was followed by a mixture of EDTA and peptide DJK-5.

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