Antibiofilm Effect of D-enantiomeric Peptide Alone and Combined with EDTA *In Vitro*

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Abstract

Introduction: The aim of this study was to evaluate the effect of DJK-5, a newly developed cationic antimicrobial peptide, on oral multispecies and *Enterococcus faecalis* biofilms alone or combined with the endodontic chelating agent EDTA *in vitro*. **Methods:** Oral multispecies biofilms from 2 donors and *E. faecalis* VP3-181 biofilm were grown on collagen-coated hydroxyapatite disks. After incubation for 3 days or 3 weeks, the biofilms were exposed to sterile saline (negative control), 8.5% EDTA, 2% chlorhexidine digluconate (CHX), 5 μg/mL DJK-5, 10 μg/mL DJK-5, a mixture of 5 μg/mL DJK-5 and 8.5% EDTA (final concentration), or a mixture of 10 μg/mL DJK-5 and 8.5% EDTA, all for 1 and 3 minutes. The proportions of dead bacteria in the biofilms were assessed by the LIVE/DEAD staining (Thermo Fisher Scientific, Waltham, MA) and confocal microscopy. **Results:** The peptide DJK-5 rapidly killed most bacteria in all biofilms, with significant differences to the control, 8.5% EDTA and 2% CHX ($P < .01$). Basically, a higher DJK-5 concentration and longer exposure (3 minutes) were more effective than a low concentration and short time exposure ($P < .05$). There were no significant differences in antibiofilm activities between DJK-5 used alone or in the mixture with 8.5% EDTA at either concentration. EDTA (8.5%) had no significant antimicrobial effect compared with the negative control ($P > .05$), but, unlike DJK-5 alone, the mixture retained the ability to remove smear layers. In peptide groups, there were no significant differences in dead bacteria proportions between 3-day and 3-week biofilms, except for 10 μg/mL DJK-5 used alone for 3 minutes on the multispecies biofilms. **Conclusions:** DJK-5 exerted antibiofilm ability on *E. faecalis* and oral multispecies biofilms grown on hydroxyapatite disks, both alone and when combined with 8.5% EDTA. *(J Endod 2017;■ :1–6)*

Key Words

Biofilm, confocal laser scanning microscopy, DJK-5, EDTA

Significance

A novel mixture of an antibiofilm peptide and EDTA has strong antibiofilm effectiveness and the ability to remove the smear layer. This solution may be used as an alternative final irrigant in endodontic treatment.

Bacterial invasion and colonization of root canal systems are the main causes of irreversible pulpitis and apical periodontitis. Microorganisms on canal wall and in dentinal tubules are organized in biofilms. Obviously, eradiating biofilms in the root canal system plays a critical role in endodontic treatment. Mechanical instrument techniques can remove much of those biofilms that are touched by the rotary files during canal preparation; however, in many other areas, such as lateral canals, fins, and isthmuses, other means are necessary in an effort to try to remove or kill the microbes (1, 2). Consequently, irrigation with solutions with tissue-dissolving and/or antimicrobial activity and the use of locally applied medicaments are needed to optimize the effect of endodontic treatment (3). Various irrigating solutions have been used across the years. Sodium hypochlorite (NaOCl), EDTA, and chlorhexidine digluconate (CHX) are the most commonly used solutions for this purpose.

Despite the different antimicrobial strategies, microbes in the biofilms may still survive, which can later lead to renewed growth of the biofilm and reinfection of the root canal system. The main reason for the difficulty to completely eradicate root canal microbes is that in the biofilm they are protected by extracellular polymeric substances and the biofilm ecology (4). Therefore, they are more resistant to disinfecting solutions and other antimicrobial strategies than planktonic bacteria (5). Consequently, new antimicrobial and antibiofilm substances and strategies are being continuously developed to improve the success of endodontic treatment.

Antimicrobial peptides, also known as host defense peptides, are natural or synthetic peptides with antimicrobial activity against many different types of bacteria in the planktonic state and/or in biofilms (6). They also act in the process of innate and/or adaptive immune modulation through recruitment and activation of immune cells, chemoattraction, regulating cell autophagy, and apoptosis, leading to increased killing of bacteria and reduced inflammation (7).

A distinct subset of antibiofilm peptides in particular has drawn attention with respect to their application in the treatment of biofilm-related infections (8). DJK-5, a cationic synthetic peptide, has shown strong antibiofilm efficacy against both

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gram-positive and gram-negative bacteria in biofilms, at various stages of biofilm maturation (9, 10). DJK-5 implements its antibiofilm activity by binding to and triggering the degradation of ppGpp, which is the stress-induced second messenger nucleotide and is important in the development of bacterial biofilms of many different species (11).

After mechanical preparation and NaOCl irrigation, dentin debris and the smear layer (comprising microcrystalline and organic particle debris) are left on the root canal wall, fins, and isthmuses, where they will act as a physical barrier to impede the penetration of irrigating solution into areas that may still harbor microorganisms (12). The smear layer likely shields the bacteria from eradication and gives them the opportunity to recover, which in some cases may lead to the failure of the treatment (13). EDTA is a strong chelating agent that can dissolve the inorganic portion of the smear layer. It is usually used as a 17% solution after finished instrumentation and NaOCl irrigation (14). EDTA alone has no or only weak antimicrobial activity (15, 16).

In recent years, several combination products have been developed in which EDTA or citric acid has been combined with other chemicals in order to add antimicrobial activity to their ability to remove the smear layer (17, 18). The added substances include detergents, CHX, and tetracycline (19). In the present study, we combined a cationic, antimicrobial peptide DJK-5 with EDTA and examined its antibacterial efficacy against biofilms formed by mixed oral plaque (multispecies biofilm) and *E. faecalis* (single species biofilm) on collagen- coated hydroxyapatite (HA) disks. The null hypothesis was that the DJK-5/EDTA combination would be as effective against biofilm bacteria as DJK-5 alone.

Materials and Methods

Peptide

Peptide DJK-5 was synthesized using solid-phase 9-fluorenylmethoxy carbonyl chemistry by GenScript (Piscataway, NJ) as previously described (11). It was purified by reverse-phase high-performance liquid chromatography to a purity at least of 95%, and the identity was confirmed by amino acid analysis. A peptide stock solution (100 μg/mL) was made by suspending the powder in deionized water.

Biofilm Model

HA disks (Clarkson Chromatography Products, Williamsport, PA) were sterilized and coated with 2 mL bovine dermal type I collagen (10 μg/mL collagen in 0.012 N HCl in deionized water) (Cohesion, Palo Alto, CA) overnight at 4°C in a 24-well tissue culture plate. Both dental plaque and *E. faecalis* VP3-181 biofilms were formed on coated HA disks. Specifically, supragingival plaque on the first or second upper molars from each of 2 healthy adult volunteers were collected and suspended in brain-heart infusion broth (BHI) (Becton Dickinson, Sparks, MD). The present study was approved by the University of British Columbia Clinical Research Ethics Committee review boards (certificate H12-02430), and written informed consent was obtained from the volunteers for collecting the plaque samples. The dispersed plaque suspension was standardized to an optical density at 405 nm of 0.1 as measured in a microplate reader (ELx808 Absorbance Reader; BioTek Instruments, Inc, Winooski, VT). Subsequently, 0.2 mL of this suspension and 1.8 mL fresh BHI were added to each well containing coated HA disks and incubated anaerobically at 37°C for either 3 days or 3 weeks.

E. faecalis VP3-181 was subcultured on BHI agar (Becton-Dickinson) plates in air at 37°C overnight (20). The bacteria were suspended in BHI broth and adjusted to an optical density at 405 nm of 0.1. Collagen-coated HA disks with *E. faecalis* were placed in an incubator (37°C) in air for the designated time periods.

Exposure of Biofilms to the Experimental Solutions

After short-term (3 days) or long-term (3 weeks) incubation under anaerobic (dental plaque biofilms) or aerobic (*E. faecalis* biofilm) conditions, biofilm-covered HA disks were rinsed with phosphate-buffered saline (pH = 7.0) (Sigma-Aldrich, St Louis, MO) for 1 minute and then exposed for 1 and 3 minutes to 8.5% EDTA, 2% CHX, or 5 μg/mL or 10 μg/mL DJK-5 solutions or a mixture containing both 8.5% EDTA and either 5 μg/mL or 10 μg/mL DJK-5. Biofilms treated with 0.85% saline were used as a negative control. A total of 3 parallel samples each with 5 scanned areas (see later) were tested for each group.

Confocal Laser Scanning Microscopic Examination

After exposure to the previously mentioned solutions, all specimens were rinsed gently in 0.85% physiological saline. They were then stained with a bacterial viability stain (LIVE/DEAD *Bac*light Kit; Thermo Fisher Scientific, Waltham, MA) and scanned with confocal laser scanning microscopy as described previously (10). Three-dimensional volume stacks were constructed with Imaris 7.2 software (Bitplane Inc, St Paul, MN), and the total volume of red (dead bacteria) and green (live bacteria) fluorescence was measured. The proportion of dead bacteria was calculated from the proportion of red fluorescence of the total of green and red fluorescence.

Smear Layer Removal

The dentin disk samples were prepared as previously described (18). Subsequently, samples were exposed to 2 mL of the following solutions: 6% NaOCl for 5 minutes; distilled water for 1 minute; and then 10 μg/mL DJK-5 + 8.5% EDTA, 17% EDTA alone, or water for 5 minutes followed by a final rinse with distilled water for 1 minute. The exposure to solutions was performed in a 20-mL beaker placed on an Orbit shaker (Lab-Line Instruments Inc, Melrose Park, IL) set at 60 rpm at room temperature. The specimens were examined for smear layer removal using scanning electron microscopy (Hitachi SU3500 VPSEM; Hitachi High-Technologies Canada Inc, Toronto, Canada) and observed at 3 kV under a magnification of 1500×.

Statistical Analysis

Statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL). One-way analysis of variance was implemented, and the post hoc Fisher least significant difference multiple comparison test was applied when necessary; significance was considered to occur at the $P < .05$ confidence level.

Results

DJK-5 peptide alone or mixed with EDTA killed bacteria effectively both in young (3 days) and old (3 weeks) biofilms (Figs. 1*A1–G2* and 2). The proportion of killed bacteria ranged from 58.9%−89.2% depending on the peptide concentration, exposure time, and biofilm age (Fig. 2). Higher peptide concentrations and a longer exposure time (3 minutes) resulted in the highest killing. Three-week-old plaque biofilms were slightly more resistant to the peptide than the 3-day old plaque biofilms ($P < .05$), but the difference was statistically significant only when 10 μg/mL DJK-5 was used alone for 3 minutes on the multispecies biofilms (Fig. 2*A1–B2*). With *E. faecalis*, differences between young and old biofilms were small and not statistically significant in all groups (Fig. 2*C1* and *C2*). Differences between the 3 biofilm groups (2 plaques, *E. faecalis*) were also small. Importantly, mixing the peptide with EDTA did not reduce the effectiveness of the peptide (Figs. 1 and 2).

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