Antibacterial Efficacy of a Human β-Defensin-3 Peptide on Multispecies Biofilms

Jin-Kyung Lee, DDS, MSD, Seok Woo Chang, DDS, PhD,[†] Hiran Perinpanayagam, DDS, PhD,[‡] Sang-Min Lim, DDS, MSD,[§] Yoon-Jeong Park, PhD,[||] Seung Hyun Han, PhD,[¶] Seung-Ho Baek, DDS, PhD,* Qiang Zhu, DDS, PhD,[#] Kwang-Shik Bae, DDS, PhD,* and Kee-Yeon Kum, DDS, PhD**

Abstract

Introduction: The aggregation of mixed bacterial flora into sessile biofilms on root canal surfaces can be one of the causes of persistent apical periodontitis. The aim of this study was to evaluate the antibacterial efficacy of human β-defensin-3 (HBD3) peptide on multispecies biofilms by using confocal laser scanning microscopy. **Methods:** *Actinomyces naeslundii, Lactobacillus salivarius, Streptococcus mutans*, and *Enterococcus faecalis* were cultured in a peptone-yeast-glucose broth, and their culture suspensions were combined in equal proportions. The mixed bacteria were inoculated on sterile coverslips placed into the wells of tissue culture plates to permit the formation of mixed species biofilm. After incubation for 3 weeks, the samples were treated for 24 hours with saline (control), saturated calcium hydroxide solution (CH), 2% chlorhexidine solution (CHX), and 50 μg/mL HBD3 solution. A commercial biofilm/viability assay kit was used to assess cell viability and analyze the 3-dimensional architecture of biofilms. The percentage of dead cells was determined from the ratio of biovolumes for the red subpopulation and the total biofilm. **Results:** Three medication groups showed a significant reduction of biovolume within the biofilms compared with the control group ($P < .001$). The HBD3-treated biofilms had a higher percentage of dead cells than the other medication groups ($P < .05$). The CH and CHX groups showed higher levels of bactericidal activity than saline ($P < .05$), and there was no significant difference between the 2 groups ($P > .05$). **Conclusions:** HBD3 peptide exhibited more antibacterial activity against mature multispecies biofilms *in vitro* than either CH or CHX. *(J Endod 2013;39:1625–1629)*

Key Words

Antimicrobial efficacy, biovolume, confocal laser scanning microscopy, dead cell/live cell ratio, human β-defensin-3 peptide, multi-species biofilms

Microbial infection of the root canal is considered to be the main cause of apical periodontitis (1). Although endodontic treatment removes the majority of bacteria, it is difficult to completely eradicate them from the root canal system (2). The aggregation of mixed bacterial flora into sessile biofilms on root canal surfaces makes them particularly difficult to remove (3).

Biofilms are communities of microorganisms with a surface embedded in an extracellular matrix of polysaccharide and proteins. Bacteria within biofilms are more resistant to antimicrobial agents compared with planktonic states (4). The minimum inhibitory concentration of bacteria growing on a surface can range from 2-fold to 1000-fold greater than when the same cells are grown planktonically (4). Their increased resistance within biofilms involves protective mechanisms that are not fully understood (5). These mechanisms may involve the changes in bacterial metabolism and genetic expression that are associated with sessile growth (6). In addition, it may be due to physical or chemical barriers within biofilms that limit antimicrobial penetration (7). Furthermore, bacteria within biofilms benefit from the establishment of a broader habitat range for growth, increased metabolic diversity and efficiency, protection against competing bacteria, host defenses, and environmental stress, and they demonstrate enhanced pathogenicity (8).

Accordingly, several intracanal medicaments have been used to disrupt biofilms and thereby eradicate residual bacterial infections within root canals. For the most commonly used intracanal medicament calcium hydroxide (CH), its antibacterial efficacy is compromised by the buffering effect of dentin (9), resistance of *Enterococcus faecalis* to the hydroxyl ion (10), and low solubility and diffusibility (11).

From the *Department of Conservative Dentistry, Dental Research Institute, Seoul National University Dental Hospital, Seoul National University School of Dentistry, Seoul, Republic of Korea; [†]Department of Conservative Dentistry, School of Dentistry, Kyung Hee University, Seoul, Republic of Korea; [‡]Division of Restorative Dentistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Canada; [§]Department of Conservative Dentistry, Jukjeon Dental Hospital, College of Dentistry, Dankook University, Jukjeon, Republic of Korea; [||]Intellectual Biointerface Engineering Center, Dental Research Institute, Seoul National University School of Dentistry, Seoul, Republic of Korea; [¶]Department of Oral Microbiology and Immunology, Dental Research Institute, Seoul National University School of Dentistry, Seoul, Republic of Korea; and [#]Division of Endodontology, Department of Oral Health and Diagnostic Sciences, School of Dental Medicine, University of Connecticut Health Center, Farmington, Connecticut.

Drs Jin-Kyung Lee and Seok Woo Chang contributed equally to this work.

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Address requests for reprints to Dr Kee-Yeon Kum, Department of Conservative Dentistry, Dental Research Institute, Seoul National University Dental Hospital, Seoul National University School of Dentistry, 275-1 Yeongun-Dong, Jongro-Gu, Seoul, South Korea. E-mail address: kum6139@snu.ac.kr

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Another commonly used agent chlorhexidine (CHX) has a broad spectrum of antimicrobial activity and good substantivity but is inactivated by physiological salts (12) and has limited penetration of the deep layer of biofilms (13).

A novel intracanal medicament human β-defensin-3 (HBD3) is a cationic antimicrobial peptide with strong antibacterial and immunoregulatory activity (14). HBD3 was reported to be induced by heat and lipopolysaccharide in human dental pulp cells and to play a role in preventing pulpitis (15). In previous studies, HBD3 was more effective than CH against anaerobes in the root canal (16) and *E. faecalis* in a dentin block infection model (17). In addition, HBD3 could neutralize the lipoteichoic acid of *E. faecalis* (18). However, the antimicrobial efficacy of HBD3 on mixed biofilms formed by endodontic pathogens has not been studied. Therefore, the aim of this study was to evaluate the antibacterial efficacy of HBD3 compared with CH and CHX on multispecies biofilms.

Materials and Methods

Bacterial Strains and Multispecies Biofilm Formation

E. faecalis (ATCC 29212), *Actinomyces naeslundii* (ATCC 12104), *Lactobacillus salivarius* (ATCC 11741), *and Streptococcus mutans* (RSHM 676), which have been reported as endodontic pathogens, were used in the present study (19, 20). The 4 strains were grown in the liquid growth medium containing peptone-yeast-glucose (PYG) in 10 mmol/L potassium phosphate-buffered saline (pH 7.5) and incubated in 5% CO_2 at 37°C overnight. Each 500-μL aliquot of culture suspension was transferred to 10 mL fresh PYG and incubated at 37°C. When the suspensions reached mid-log growth phase, the 4 bacteria were mixed in equal proportions.

A sterile coverslip was placed into each well of a 12-well tissue culture plate (Costar, Corning, NY). Two hundred microliters of bacterial suspension and 2.8 mL PYG broth were transferred to each well for the formation of the mixed species biofilm. The broth was changed every 2 or 3 days for 21 days.

Antimicrobial Treatment on Multispecies Biofilms

After incubation at 37°C for 3 weeks, the broth was aspirated aseptically from each well. Then, 0.9% saline was added for 3 minutes to remove unattached bacteria. The biofilm samples were treated for 24 hours with 3 mL of one of the following:

1. 0.9% sterile saline
2. Saturated CH solution (DC Chemical Co Ltd, Seoul, South Korea)
3. 50 μg/mL HBD3 solution (NIBEC, Seoul, South Korea)
4. 2% CHX digluconate (prepared freshly from 20% stock solution; Sigma-Aldrich, St Louis, MO)

The experimental concentration of HBD3 (50 μg/mL) was determined from a preliminary study. After the exposure time, each sample was washed gently with saline.

Bacterial Cell Staining for Confocal Scanning Laser Microscopy

After treatments with the tested medicaments, the FilmTracer LIVE/DEAD Biofilm Viability Kit (Molecular Probes, Carlsbad, CA) containing SYTO 9 and propidium iodide was used to stain live and dead bacteria in the biofilms, according to the manufacturer's instructions. Bacteria with intact cell membranes stain fluorescent green by SYTO 9, whereas bacteria with damaged membranes stain red by propidium iodide. The specimens were observed immediately by using a confocal scanning laser microscope (CLSM) (LSM700; Carl Zeiss, Jena, Germany) with the ×40 lens. Images were acquired with software ZEN 2010 (Carl Zeiss) at a resolution of 512 × 512 pixels and with a zoom factor of 2.0. Individual biofilm images covered an area of 80 by 80 μm. Three randomly selected areas were imaged in each biofilm sample, and a total of 3 samples were observed per group. In all cases, the z step for images in a stack was 1 μm, and there were 15 stacks.

Image Analysis of Biofilms and Statistical Analysis

CLSM images were analyzed by using the software bioImage_L (http://bioimagel.com). The percentage of dead cells was obtained by calculating the percentage of the biovolume of the red subpopulation from total biovolume of the biofilm. The results were subjected to one-way analysis of variance and a post hoc test at a significance level of $P < .05$ (SPSS version 12.0, SPSS for Windows; SPSS Inc, Chicago, IL).

Results

Antimicrobial Treatment Reduced Biofilm Biovolumes

The treated biofilms had significantly ($P < .001$) less biovolume than the untreated controls (Fig. 1). The biofilms that had been treated with CH, HBD3, or CHX had significantly ($P < .001$) reduced biovolumes compared with the saline controls. However, there was no significant ($P > .05$) difference in the reduced biovolume between the CH-, HBD3-, or CHX-treated biofilms.

In saline controls, the biomass for the total population of bacterial cells appeared to be normally distributed across the z-level plot (0–14 μm), with the highest density at around 6 μm (Fig. 2*A*). However, in the treated biofilms, the biomass of the total population was largely restricted to the deeper layers (1–6 μm), and the highest densities were at 2–4 μm (Fig. 2*B–D*). This skewed distribution of biomass was similar for all of the treated biofilms, except that live (green) cells made up the biovolumes in the CH-treated and CHX-treated biofilms, and dead (red) cells accounted for the HBD3 treatments.

HBD3 Was More Bactericidal on Biofilms

The HBD3-treated biofilm were largely composed of dead (red) cells (Fig. 3*C*), whereas the CH-treated and CHX-treated biofilms contained a mix of live (green) and dead cells (Fig. 3*B* and *D*), and the saline controls contained only live cells (Fig. 3*A*). The proportion of dead cells in the HBD3-treated biofilms was significantly

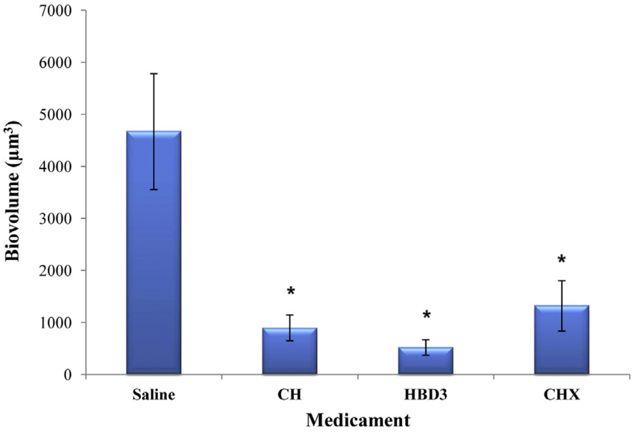

Figure 1. Effect of intracanal medicaments on biovolume (μm³) of multispecies biofilms. Biofilm samples composed of 4 bacterial strains were incubated for 3 weeks. Biofilms were treated for 24 hours with each medication and stained with FilmTracer LIVE/DEAD Biofilm Viability Kit. The values of biovolume were calculated by the software bioImage_L. *Significant difference between control (saline) group and the other 3 medicaments.