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Antibacterial efficacy of a human β-defensin-3 peptide using dentin infection model and biofilm viability assay

상아질 감염 모델 및 바이오필름 활성 검사를 이용한 human β-defensin-3 peptide의 항균 효과에 대한 연구

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of a human ß-defensin-3-derived peptide

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Abstract

Antibacterial efficacy of a human β-defensin-3-derived peptide using dentin infection model and biofilm viability assay

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The aggregation of 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 compared with calcium hydroxide (CH) and chlorhexidine (CHX) on *Enterococcus faecalis (E. faecalis)*-infected dentin blocks and multi-species biofilms.

Standardized human dentin blocks were infected with *E. faecalis* ATCC 29212 for 3 weeks. Aqueous CH paste, a 2% CHX gel, an HBD3 peptide gel, control peptide (CP) and saline were treated in canal lumen. 12 dentin block samples were included in each group. After 1 week of medication, the dentinal samples at the depth of 200 and 400 μm were collected from medicated canal lumens. Bacterial growth was assessed by spectrophotometric analysis of optical density (OD) after 72 hours of incubation. Repeated-measures analysis of variance and Tukey's *post hoc* test were used for data analysis.

For multispecies biofilm formation, *Actinomyces naeslundii, Lactobacillus salivarius, Streptococcus mutans,* and *E. faecalis* were cultured in a peptone-yeast-glucose broth and their culture suspensions combined in equal proportions. The mixed bacteria were inoculated on sterile cover slips placed into the wells of tissue culture plates. After incubation for three weeks, the samples were treated for 24 hours with saline, saturated CH solution, 2% CHX solution, and 50 μg/ml HBD3 solution. The percentage of dead cells was determined by viability staining and confocal laser scanning microscopy. The results were subjected to one-way analysis of variance and a post hoc test.

In dentin block study, the HBD3 group was associated with significantly lower OD values ($p < 0.05$) than the CH or CHX groups at both depths. The CH group did not differ significantly from CP or saline group at either depth ($p > 0.05$). There was no significant difference ($p > 0.05$) in the OD values of the inner (200 μm) and outer (400 μm) dentinal samples for any group.

In multispecies biofilm study, three medication groups showed a significant reduction of biovolume within the biofilms compared with the control group ($p < 0.05$). The HBD3-treated biofilms had a higher percentage of dead cells than the other medication groups ($p < 0.05$). The CH and CHX showed higher levels of bactericidal activity than saline ($p < 0.05$), and no significant difference between the two groups ($p > 0.05$).

HBD3 exhibited marked antibacterial activity against *E. faecalis* biofilms in dentin block and multi-species biofilms, than either CH or CHX.

Key words: antimicrobial efficacy, human β-defensin-3 peptide, dentin block, optical density, multi-species biofilms, confocal laser scanning microscopy.

Contents

Antibacterial efficacy of a human β-defensin-3-derived peptide using dentin infection model and biofilm viability assay

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Ⅰ. Introduction

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 more difficult to remove by routine endodontic procedure[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 two- 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].

Additionally, 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 (E. faecalis)* to the hydroxyl ion[10], and low solubility and diffusibility[11]. 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]. Therefore, alternative intracanal medicaments should be explored to maximize the eradication and biofilm disruption of the therapy-resistant flora.

A human β-defensin-3 (HBD3) is a cationic antimicrobial peptide with strong antibacterial and immunoregulatory activity[14]. Human β-defensin 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 *in vitro*[16]. In addition, HBD3 could neutralize the lipoteichoic acid (LTA) of *E. faecalis*[17]. However, the antimicrobial efficacy of HBD3 on biofilms formed by endodontic pathogens has not yet been studied. The aim of this study was to evaluate the antibacterial efficacy of HBD3 compared with CH and CHX on *E. faecalis*-infected dentin blocks and multi-species biofilms.

Ⅱ. Theoretical background

Inter-appointment intracanal medication can be used to further reduce remaining bacteria in the root canal system after chemomechanical preparation[18], and to prevent re-infection as a chemical and physical barrier in cases of multiple visits[19].

CH is one of the most commonly used intracanal medicaments since the 1920s[20]. The antimicrobial activity of CH is due to the release and diffusion of hydroxyl ions (OH^-) leading to a highly alkaline environment (pH 12.5-12.8) which prevents the survival of micro-organisms[19]. CH also inactivates LPS and can assist periapical tissue repair[11]. But, its efficacy is inhibited by dentin protein buffering that limits the pH to reach the critical level for killing the bacteria in the infected root canal[21] and diluted by tissue fluid as time goes in the root canal[12]. Furthermore, there has been increasing concern about the insufficient antimicrobial efficacy of CH against *E. faecalis* even after prolonged contact between the medication and root canal[22-24]. This might be due to the deep invasion of *E. faecalis* into the dentinal tubules[25] and the ability to maintain pH homeostasis by a function proton pump[26]. Many studies indicate that *E. faecalis* may be existed in root-filled teeth with persistant periodontitis with the prevalence of this species ranging from 12 to 70 percent[27]. Thus, the use of CH as the intracanal medicament in the retreatment case with apical periodontitis has been questioned[28, 29].

CHX is a broad-spectrum antimicrobial agent whose positively charged molecules can be adsorbed onto dentin and subsequently released from dental tissues, resulting in antimicrobial substantivity[11, 30]. CHX was more effective than CH in eliminating *E. faecalis* from inside dentinal tubules[31]. Its acting mechanism is based on the interaction between the positive charge of CHX and negatively charged phosphate groups on the bacterial cell wall. This increases the permeability of the cell wall which allows the CHX molecule to penetrate into the bacteria with

intracellular toxic effects[32]. However, CHX also showed a few shortcomings for general use of endodontic medications. It was reported to be not effective for killing microbes in mature multispecies biofilms[33]. In addition, CHX may be inactivated or buffered by dentin and other substances present in root canals[12]. CHX cannot dissolve organic substances and its routine application requires attention when NaOCl is used as a root canal irrigant. Mixing of NaOCl and CHX produce a cytotoxic brown precipitate that stains the walls of the pulp chamber and is difficult to remove[11].

To compensate these shortcomings, various attempts have been made. Alternative intracanal medications such as antibiotics and iodine potassium iodide were introduced[19], but problems such as appearance of resistant strains and tooth discoloration were revealed. The combinations of several medicaments were expected to provide a more potent effect[34, 35]. However, no medicaments have been found to be completely predictble in its efficacy.

Defensins are a family of antimicrobial peptides that have a molecular weight of 4–5 kDa and typically expressed in mammalians, insects and plants[36]. Defensins contribute to host immune response by direct bacteriocidal activity, as well as effector and regulatory functions. The family is subdivided into α-, β- and θ-defensins, only α- and β-defensins are expressed in humans[37]. Human defensins contain a conserved motif of six disulfide-linked cysteines forming three disulfide bonds. To date, six human α-defensins (HNP-1–4, HD-5 and HD-6) and eleven human β-defensins (HBD-1–6 and HBD-25–29) have been discovered[37].

Defensins are implicated in a wide variety of medical conditions and diseases. In many cases, a disease state is accompanied by a change in the amount of defensin expression in the diseased tissue. An impaired production may lead to an increased

colonization by infectious agents, but in high amounts the defensins might also exaggerate inflammatory responses and lead to disease[14]. Crohn's colitis is associated with an impaired induction of HBD2 and HBD3, whereas active ulcerative colitis patients show enhanced expression of both HBD2 and HBD3[38] was also related to skin diseases such as psoriasis and atopic dermatitis[39], vascular diseases[40], several pulmonary disorders[41, 42].

The pharmaceutical potential of human defensins seems to be high considering their strong and broad-spectrum antimicrobial activity[43]. Moreover, the inducible expression of human defensins could provide a new approach for the treatment of infections. It might be possible to develop derivatives that can induce in vivo production of defensins, avoiding side effects by synthetic analogues and the difficulty of delivering them to the desired site of action[44]. Especially, gene therapy is a promising method with a great possibility for the treatment of pathologies with impaired production of antimicrobial peptides, like periodontal disease, Crohn's disease, and cystic fibrosis[36]. The misuse of existing antibiotics has resulted in bacterial resistance to them. Human defensins might be ideal therapeutic agents to avoid the problems of acquired resistance[44].

Peptides based on a defensin template have not been investigated in clinical studies so far. The high cost of manufacturing the whole peptide is the principal problem preventing the clinical use. Approach to reduce cost is to use conventional or solution-phase peptide synthesis to decrease the size of peptides as in present study[45]. To date, several α-helical and defense peptides have entered clinical trials, but none have been approved for medical use yet. Several derivatives of peptides originated from animals demonstrated efficacy in phase III trials, but failed to achieve approval by the US Food and Drug Administration, since no advantage could be demonstrated over existing therapies[46, 47].

Among several human defensins, HBD3 have a broad spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi and some viruses[14] and is widely expressed in oral tissues[48]. Furthermore, HBD3 can maintain its broad spectrum of activity in the presence of physiological saline unlike other human defensins that are salt sensitive[43]. It has been demonstrated that HBD3 is frequently expressed in gingival epithelia and play a protective role in the maintenance of periodontal health[49] and exerts a strong bacteriocidal effect against various periodontal pathogens and cariogenic bacteria suggesting a potential therapeutic use of HBD3 in the local treatment of oral infections[50]. Dale et al. reported that the presence of antimicrobial peptides such as HBD3 in saliva might be a biological factor that contributes to resistance to caries[51].

In previous studies against endodontic pathogens, recombinant HBD3 significantly eliminated pathogenic bacteria in root canals and showed the potential to apply endodontic disinfectants[16]. In addition, HBD3 could neutralize the lipoteichoic acid (LTA) of *E. faecalis*[17]. However, little can be found in the literature about the antimicrobial efficacy of HBD3 against bacteria in root canal lumen and multi-species biofilms formed by endodontic pathogens.

Ⅲ. Materials and Methods

1. Antimicrobial experiment using *E. faecalis*-infected dentin blocks

1) Preparation of dentin blocks

This study was carried out under the approval of the Institutional Review Board of Seoul National University Dental Hospital, Seoul, Korea (IRB # ERI12002).

Seventy-two single-rooted human premolar teeth with fully formed apices and caries-free crowns were collected from patients undergoing tooth extraction for orthodontic and periodontic reasons in the Department of Oral and Maxillofacial Surgery. Calculus and soft tissues on root surface were cleaned with an ultrasonic scaler and stored in a 0.5% sodium azide solution at 4°C. The model proposed by Haapasalo & Ørstavik (1987) was adopted and modified for this study[25]. The middle one-third of each root was sliced into a 6-mm-thick section with an Isomet precision saw (Bueher, Lake Bluff, IL, USA). The cementum was removed from the root surface, and the entire root canal was enlarged using a size 2 Gates-Glidden drill (0.7 mm in diameter; JS Dental Manufacturing Inc., Ridgefield, CT, USA) in a low-speed handpiece. The smear layer was removed by immersing the specimens in an ultrasonic bath of 17% EDTA (pH 7.2) followed by 2.5% NaOCl, each for 5 minutes. The NaOCl was then neutralized with a 5% sodium thiosulfate solution for 5 minutes, and the dentin blocks were washed three times in distilled water to remove residual disinfectants. The dentin blocks were sterilized by autoclaving for 15 minutes at 121°C. After sterilization, the dentin blocks were incubated in brain heart infusion (BHI, BD Biosciences, Franklin, NJ, USA) at 37 °C for 24 hours to ensure no bacterial contamination (Fig. 1).

2) Preparation of HBD3 peptide gel

The HBD3 peptide (NIBEC, Seoul, Korea) was prepared by F-moc-based chemical solid-phase synthesis and were composed of 15 amino acids. As a control peptide, the mis-matched sequence of the HBD3 peptide was prepared using a same method. To assess the bactericidal activity of HBD3 against *E. faecalis*, the minimum inhibitory concentration (MIC) and the minimum

bactericidal concentration (MBC) were determined by using the microdilution method[43]. Briefly, 1 x 10^7 colony-forming units (CFU) of *E. feacalis* (ATCC 29212) in midlog growth phase were suspended with 10 mmol/L sodium phosphate buffer (pH 7.4) and inoculated into 96-well polystyrene plates by 50 µL. Various concentrations of HBD3 (50 µL) were added into bacterial suspensions and incubated for 3 hours at 37°C. The antimicrobial activity of HBD3 was analysed by plating serial dilutions of the incubation mixture on BHI agar and determination of the CFU for the following day. Both positive and negative controls consisting of either bacterial suspension or broth alone were included in all experiments. The experiments were performed three times in triplicate.

3) Infection of dentin blocks

Sterile dentin blocks were placed in conical tubes containing fresh BHI (10 mL). *E. faecalis* grown overnight in BHI broth and adjusted to the 0.5 turbidity reading of the McFarland scale (1.5 x 10^8 CFU/mL) was inoculated into each root canal. The tubes were incubated for 3 weeks at 37°C in a humidified incubator with 5% CO_2. Fresh broth was replaced every second day. The turbidity of the medium during the incubation period indicated bacterial growth. The purity of the cultures was confirmed by Gram staining, catalase production and colony morphology on BHI blood agar and by the use of a biochemical identification kit (API 20 Strep system; bioMérieux SA, Marcy l'Etoile, France). Bacterial penetration into the dentinal tubules and biofilm formation was confirmed using scanning electron microscopy (Fig. 2).

4) Antimicrobial treatment

Following the infection period, the dentin blocks were removed from the test tube. The root canal was rinsed thoroughly with a sterile saline solution and dried with sterile paper points. The outer surfaces of dentin blocks were coated with two layers of nail varnish, and all specimens were fixed to the bottom of a sterile petri dish with decontaminated paraffin wax. The specimens were divided randomly into six groups, and the root canals were treated as follows: 1) CH group - CH (DC Chemical Co. Ltd., Seoul, Korea) mixed with distilled water was placed by means of a Lentulo-spiral (Dentsply Maillefer, Ballaigues, Switzerland) into the canal lumen until full. 2) CHX group - 2% CHX gel (Itapetin-inga, São Paulo, Brazil) was placed into the lumen by injection until full. 3) saline group – sterile saline was placed into the lumen by injection until full. 4) HBD3 group – the MBC was used as the concentration of HBD3 peptide. Peptide gels were prepared as follows. A 20% polyvinylpyrrolidone (PVP) solution was autoclaved for 1 h at 121 °C and then used as the gel base. The HBD3 peptide mixed with the 20% PVP solution and placed into the lumen by injection with a sterilized syringe until full. 5) As a control, gel containing a control peptide of HBD3 (CP, same concentration of MBC) was placed into the canal lumen by injection until full. Uninfected sterilized dentin blocks (US) were also included as a control for confirmation of the aseptic procedure of the experiment. 12 dentin block samples were included in each group. After intracanal medication, the blocks were sealed coronally with decontaminated paraffin wax and incubated in an anaerobic environment at 37°C for 1 week in a humidified incubator with 5% CO_2. Once placed, the medicaments were not replenished.

5) Measurement of antibacterial activity

After 1 week of root canal medication, each canal was washed with 10 mL of sterile saline to remove the intracanal medicaments and dried with sterile paper points. Dentin chips were harvested from the canal lumens with the sequential use of size 90 (200-μm depth, inner dentin) and size 110 (400-μm depth, outer dentin) sterilized files (LightSpeed; Discus Dental, Culver, CA, USA). The dentin chips were immediately placed into separate test tubes containing 3 mL of fresh BHI broth plus a neutralizer to avoid continued action of the medicaments. The neutralizer for CH was 0.5% citric acid, whilst 0.5% Tween 80 + 0.07% lecithin was used for CHX[52]. The neutralizer for the HBD3 peptide was 0.1 mg/mL of cathepsin[53]. The tubes were then incubated at 37°C for 72 hours in a humidified incubator with 5% CO_2 to allow the growth of any bacteria harboured in the dentine and observed for microbial growth visualized by medium turbidity. When turbidity was detected, bacteria were identified to ensure that there was no contamination other than *E. faecalis* by Gram staining, catalase production and colony morphology on BHI blood agar and by the use of a biochemical identification kit (API 20 Strep system; bioMérieux SA). Then, the optical density (OD) of the broth, which is directly related to the presence of bacteria, was read using a spectrophotometer (DynaTech Laboratories, Billingshurst, UK) at 530 nm. The OD readings were adjusted to the OD value of fresh, sterile BHI broth as a baseline.

6) Data analysis

Within each group, the mean OD values at the inner and outer depths were compared using a paired *t*-test. Repeated-measures analysis of variance was used to analyze the differences between the medication groups by adjusting the two correlated measurements from each canal. Tukey's *post hoc* test was then used to

compare the 200- and 400-μm depths of the dentinal samples from the individual groups separately. The data were statistically analyzed using SPSS version 12 (SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was considered significant.

2. Antimicrobial experiment on multi-species biofilms

1) Bacterial strains and multi-species 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[54, 55]. The four strains were grown in the liquid growth medium containing peptone-yeast-glucose (PYG) in 10 mM potassium phosphate-buffered saline (PBS, pH = 7.5) and incubated in 5% CO_2 at 37℃ overnight. Each 500μL aliquot of culture suspension was transferred to 10 mL fresh PYG and incubated at 37℃. When the suspensions reached midlog growth phase the four bacteria were mixed in equal proportions.

A sterile cover slip was placed into each well of a 12-well tissue culture plate (Costar, Corning, NY, USA). 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 two or three days for 21 days (Fig. 3).

2) Antimicrobial treatment on multi-species biofilms

After incubation at 37℃ 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 either, (i) 0.9% sterile saline, (ii) saturated CH solution (DC Chemical Co. Ltd.), (iii) 50 μg/ml HBD3 solution (NIBEC), or (iv) 2% chlorhexidine digluconate (prepared freshly

from 20% stock solution; Sigma Chemical Co, St Louis, MO, USA). A saturated CH solution was prepared by dissolving CH powder overnight in de-ionized water[10]. 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.

3) Bacterial cell staining for confocal scanning laser microscopy (CLSM)

After treatments with the tested medicaments, the FilmTracer™ LIVE/DEAD Biofilm Viability Kit (Molecular Probes, Carlsbad, CA, USA) containing SYTO 9 and propidium iodide (PI) 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 PI. The specimens were observed immediately using a CLSM (LSM700, Carl Zeiss, Jena, Germany) with the 40 × lense. Images were acquired with software ZEN 2010 (Carl Zeiss) at a resolution of 512 x 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 three samples were observed per group. In all cases, the z step for images in a stack was 1μm, and 15 stacks.

4) 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 $p < 0.05$ (SPSS version 12.0).

Ⅳ. Results

1. Antimicrobial efficacy on *E. faecalis*-infected dentin blocks

The MIC and MBC of HBD3 against *E. faecalis* were 1 and 15.6 μg/mL, respectively (Fig. 4). HBD3 showed complete inhibition of *E. faecalis* at 15.6 μg/mL. The mean and standard deviation of OD values from the inner and outer depths of the dentinal samples of all groups are shown in Fig. 5. The OD values of HBD3 group were significantly lower than those of the other experimental groups at both depths ($p < 0.05$). The CHX group showed significantly lower OD values than the CH, saline and CP group at both depths ($p < 0.05$). The OD values of CH, saline, and CP groups did not differ significantly at either depth ($p > 0.05$). There was no significant difference in the OD values of the inner and outer dentinal samples for any group ($p > 0.05$). SN (sterilized but noninoculated) group did not show any turbidity.

2. Antimicrobial efficacy on multi-species biofilms

The biofilms that had been treated with CH, HBD3, or CHX had significantly ($p < 0.05$) reduced biovolumes compared to the saline controls (Fig. 6). However, there was no significant ($p > 0.05$) 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. 7A). 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 around 3/4 μm (Figs. 7B, 7C, and 7D). 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 and CHX-treated biofilms, and dead (red) cells accounted for the HBD3 treatments.

The HBD3 treated biofilm were largely composed of dead (red) cells (Fig. 8C), whereas the CH and CHX treated biofilms contained a mix of live (green) and dead cells (Figs. 8B and 8D), and the saline controls contained only live cells (Fig. 8A). The proportion of dead cells in the HBD3 treated biofilms was significantly ($p < 0.05$) higher than in the CH and CHX treatment and saline controls (Table 1). The proportion of dead cells in the CH and CHX treated biofilms were significantly ($p < 0.05$) higher than in the saline controls, but not significantly ($p > 0.05$) different for either treatment.

Ⅴ. Discussion

In dentin block study, the determination of OD value as an indicator of the antimicrobial effect is a rapid and convenient method, but it does not reveal viability of bacteria in the broth. Also, the use of OD values might have had some effects on the results as variation in the early log phase of growth may result in variations in the final readings after the long (72 hours) growth period[30]. To compensate these limitations, the study using fluorescent staining and CLSM was carried out, thus biovolume and dead cell to live cell ratio were also the indicators of antimicrobial effectiveness. Biovolume represents the remaining biofilm structure after the antimicrobial action of medications. Dead cell ratio was obtained as a percentage of the red volume to the combined red and green volumes, and

shows the bacterial killing ability of medicaments. The OD value in the present study showed that HBD3 peptide had significantly better antibacterial activity against *E. faecalis* in the root canal compared with CH or CHX. No significant difference in biovolume among medications suggested that CH, CHX and HBD3 possessed similar ability to disrupt biofilm structure. But dead cell ratio of HBD3 was higher than that of CH or CHX so that HBD3 had remarkable ability to penetrate and kill bacteria compared with CH and CHX. Additionally, biofilm structure and cell distribution at varying depth were visualized by z-axis scans by CLSM. The cells in the upper levels closer to the surface were more affected by exposure to the medication and the loss of biovolume.

The antibacterial efficacy of HBD3 on endodontic pathogens has been reported in previous studies[16, 17]. The mechanisms of action for HBD3 involve ionic interactions with the bacterial cell surface and membrane permeabilization[14] that are similar to that of CHX. However, HBD3 showed more antibacterial efficacy than CHX in the present study, suggesting that HBD3 may have additional modes of action against bacteria. Indeed, Zhu et al. found that HBD3 not only inhibits biofilm formation and maturation, but also reduced the preexisting biofilm on implant surfaces by inhibiting polysaccharide synthesis at the level of gene transcription[56]. Additionally, the spatial structure of N- and C-terminal fragments and the β-sheet and disulfide bonds of HBD3 have complex physiological functions that are important components of its antimicrobial activity[57]. Therefore, more research needs to be performed to fully understand the mechanism s of action of HBD3.

The CH showed limited effectiveness in killing bacteria in dentinal tubule and biofilms in this study, which is consistent with previous reports[58-60]. This might be due to the deeper invasion of *E. faecalis* into the dentinal tubules[61], the dentin

buffering effect[9], as well as the ability of *E. faecalis* to maintain pH homoeostasis by a functional proton pump[62, 63]. To be effective against bacteria located inside the dentinal tubules, the hydroxide ions need to diffuse into the dentine at sufficient concentrations to overcome the dentine buffering ability, thus reaching a sufficiently high pH to destroy or inactivate the microorganisms. Because the diffusion of hydroxide ions takes at least 3–4 weeks to reach the peak pH levels and to stabilize at these levels in the outer root dentine[64], CH might result in the limited antimicrobial effect in this study. In addition, the extracellular structure of biofilms such as exopolysaccharide matrix could inhibit diffusion so that hydroxyl ions do not reach a sufficient concentration to kill bacteria[7].

CHX was associated with significantly better antibacterial efficacy against *E. faecalis* biofilms than the CH group, which is consistent with previous findings[11, 30, 65]. This might be due to the substantial antimicrobial activity of CHX within root dentin[30, 66], whereas CH does not impart the substantial antimicrobial activity within root dentin[31]. But, CHX showed no significant difference from CH in antibacterial efficacy against multispecies biofilms. Likewise, the antibacterial effects of CHX were reduced in several studies that used mature biofilms[13, 67, 68]. This could be explained by the neutralizing effects of extracelluar polysaccharide and dead cells in the biofilms, and the limited penetration of CHX to deeper layers in biofilms[69]. It was reported that CHX had only a superficial bactericidal effect on undisturbed biofilms with a thickness of less than 65μm[70], like those in this study. Furthermore, the addition of surface modifiers to breakdown matrix, enhanced the antibacterial effectiveness of CHX[67].

HBD3 was known to have strong antimicrobial activity and also anti-endotoxin activity on the bacterial LTA and lipopolysaccharide[17, 43, 71, 72]. However, the therapeutic application of the whole protein of HBD3 has limitations because of the

weak stability of the native protein and its short half-life. In this regard, a biomimetic approach using modified (synthetic) peptide has provided advantages, including having similar efficacy as the parent molecule whilst overcoming the drawback of its short half- life[73]. So, the present study used the synthetic HBD3 peptide gel with modified sequence. The HBD3 peptide gel has several advantages as an intracanal medicament. The viscosity of the gel keeps the antimicrobial peptide in contact with root canal walls and dentinal tubules and allows the peptide to fill inaccessible canal spaces. The incidence of bacteria resistant to HBD3 peptide is rare because of its non-specific membrane-permeating mechanism[74]. The antimicrobial efficacy and range of target bacteria can be modulated by designing analogs of HBD3[14]. HBD3 also has very low cytotoxicity against host cells[75]. The cell membranes of bacteria are rich in acidic phospholipids, which are negatively charged, whereas the outer surfaces of human cell membranes are composed of lipids without any net charge. Therefore, the antimicrobial peptides will preferentially interact with the bacterial membrane by electrostatic interaction[76]. Because excellent antibacterial activity and low cytotoxicity of HBD3 seem to be advantageous in revascularization procedure, study about the applicability to immature permanent teeth is required. Furthermore, the peptide could create synergistic antimicrobial activity when incorporated with other local disinfectants such as antibiotics or CHX[50]. Further research is planned to improve antimicrobial efficacy of HBD3 by additional combination of CHX or other agents.

In this study, dentin samples were obtained from two depths by sequential drilling from the inner dentinal wall. This means that the second, deeper cut might have been contaminated from the first cut and might not actually represent a pure deeper layer[77]. A further study may be necessary to drill from the outside of the root block to obtain dentinal samples from a deeper layer. Although the 3-week incubation

period allowed the suspension of microorganism to diffuse throughout the root canal system[31], the present dentine infection method by in vitro culturing and inoculation might not simulate dense, deep and homogenous penetration of bacteria into all dentinal tubules in all specimens. Additional use of centrifugation after inoculation would help create a heavy, evenly distributed dentinal tubule infection[78].

In the present study, we used three week-old biofilms because most endodontic infections that involve biofilms are chronic infections progressing over several weeks or more. Shen et al. demonstrated that mature biofilms incubated for three weeks or longer were more resistant than planktonic bacteria and young biofilm (less than two weeks) [67]. These results could be due to the reduced metabolic activity of cells embedded in the aged biofilm[79]. However, very mature biofilms that had been incubated for more than six weeks had a resistance to CHX that was stable and similar to that of three weeks-old biofilm[67]. Furthermore, the increase of biofilm resistance to disinfecting agents is achieved between two and three weeks regardless of biofilm composition and the type of agents[80].

Various substrates have been used for biofilm formation in vitro, including human dentin, bovine dentin, cellulose acetate membranes, glass slides, and hydroxyapatite[81]. Cover slips used as substrates for biofilm in present study are readily available and inexpensive. They have a standardized shape and even surface that make it possible to grow biofilms with consistent characteristics[59]. But, they have limitations for reflecting complex in vivo conditions. Further studies are planned using dentin as substrates to mimic the root canal environment. Moreover, additional animal and clinical studies are needed to replicate these findings under in vivo conditions.

Ⅵ. Conclusion

1. HBD3 exhibited marked antibacterial activity against *E. faecalis* biofilms in dentin block than either CH or CHX.
2. CH, CHX and HBD3 showed no significant difference in ability to disrupt biofilm structure.
3. HBD3 had significant antibacterial activity against mature multi-species biofilms in vitro than CH or CHX.

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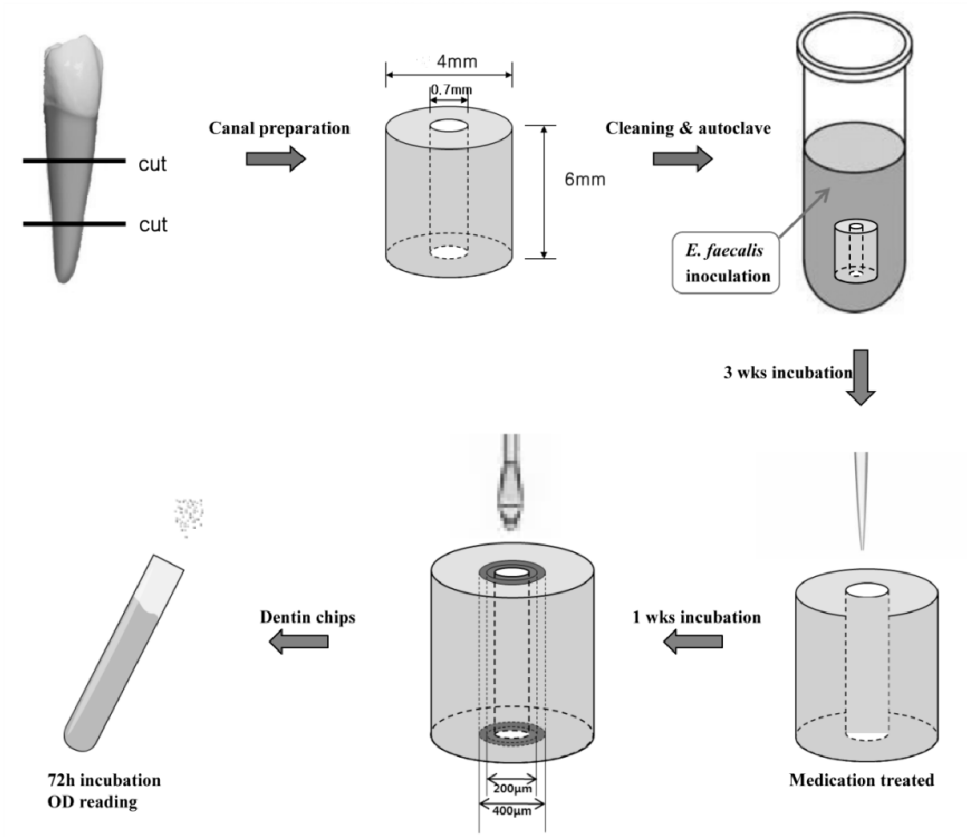

Figure 1. The procedure of antimicrobial experiment using *E. faecalis*-infected dentin blocks. Standardized human dentine blocks were infected with *E. faecalis* for 3 weeks. 4 intracanal medications were applied into canal lumen and sealed. After 1 week of medication, the dentinal samples at the depth of 200 and 400 μm were collected from medicated canal lumens. Bacterial growth was assessed by spectrophotometric analysis of optical density (OD) after 72 hours of incubation.

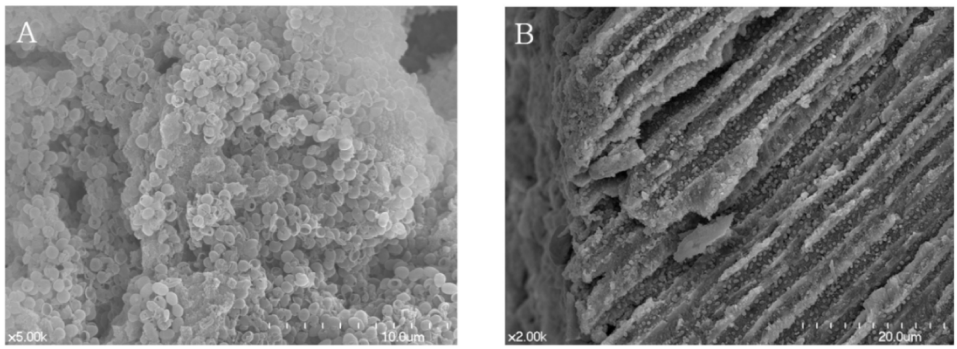

Figure 2. Scanning electron microscopic view showing the aggregation of bacteria and biofilm formation around orifices of dentinal tubules (A, 500 x) and deep penetration of *E. faecalis* into dentinal tubules after 3 weeks-infection periods (B, 200 x).

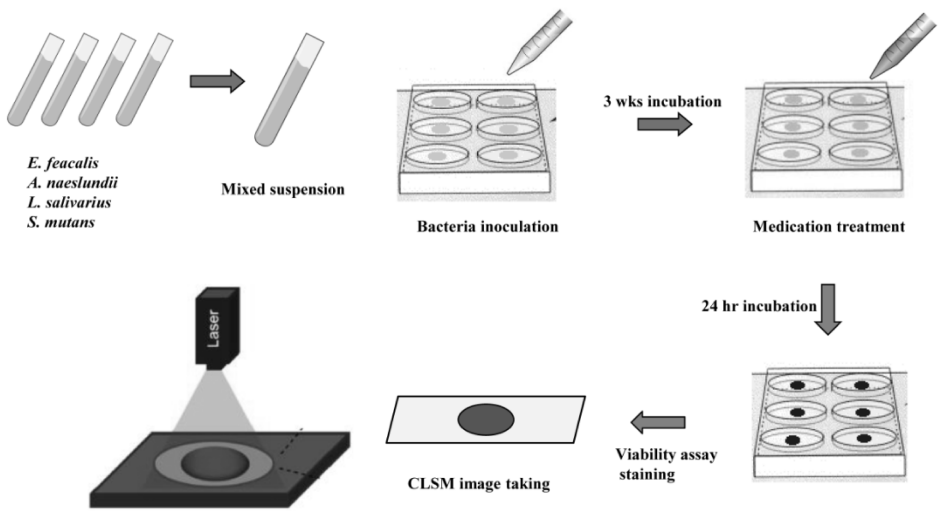

Figure 3. The procedure of antimicrobial study on multi-species biofilms. 4 bacterial strains were cultured in a peptone-yeast-glucose broth and their culture suspensions combined in equal proportions. The mixed bacteria were inoculated on sterile cover slips placed into the wells of tissue culture plates. After incubation for three weeks, the samples were treated for 24 hours with medications. A commercial biofilm/viability assay kit was used for cell staining and CLSM images were acquired.

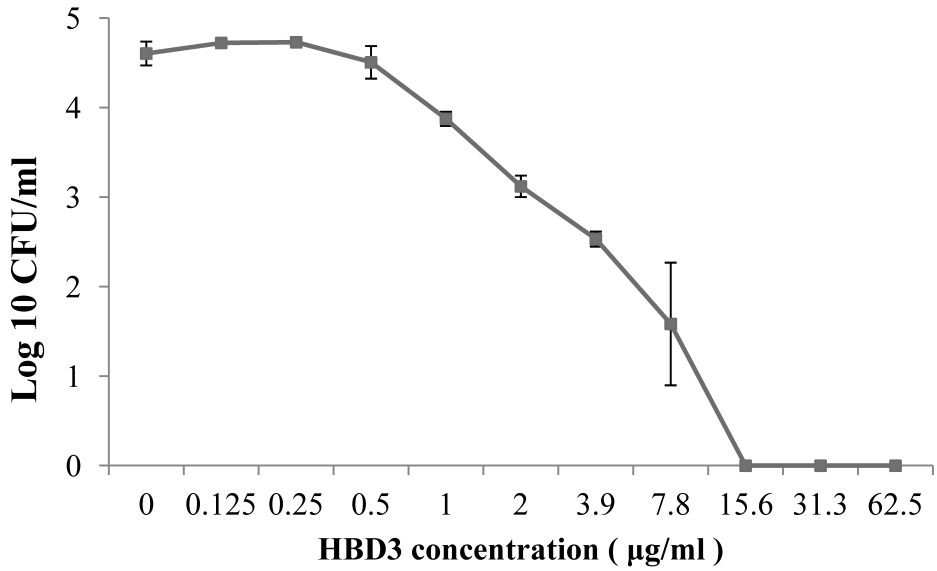

Figure 4. Antimicrobial activity of HBD3 peptide against *E. faecalis* using microdilution method. *E. faecalis* was cultured in the various concentrations of HBD3, and the minimum bactericidal concentration (15.6 μg/mL) was determined as the concentration of HBD3 peptide.

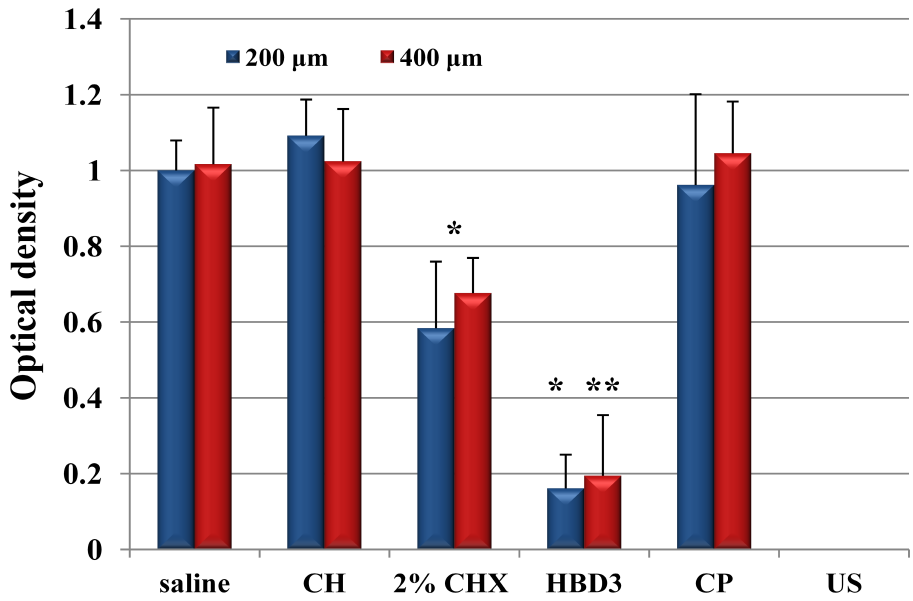

Figure 5. Optical density values of the inner (200 µm) and outer (400 µm) dentinal samples obtained from six experimental groups. CH, calcium hydroxide; 2% CHX, 2% chlorhexidine gel; HBD3, human β-defensin-3 peptide gel; CP, control peptide gel; Saline, sterile saline, US, uninfected sterilized group. An asterisk (*) indicates a statistically significant difference from the saline, CH, and CP groups ($P < 0.05$), and double asterisks (**) indicates a statistically significant difference from the CHX group ($P < 0.05$).

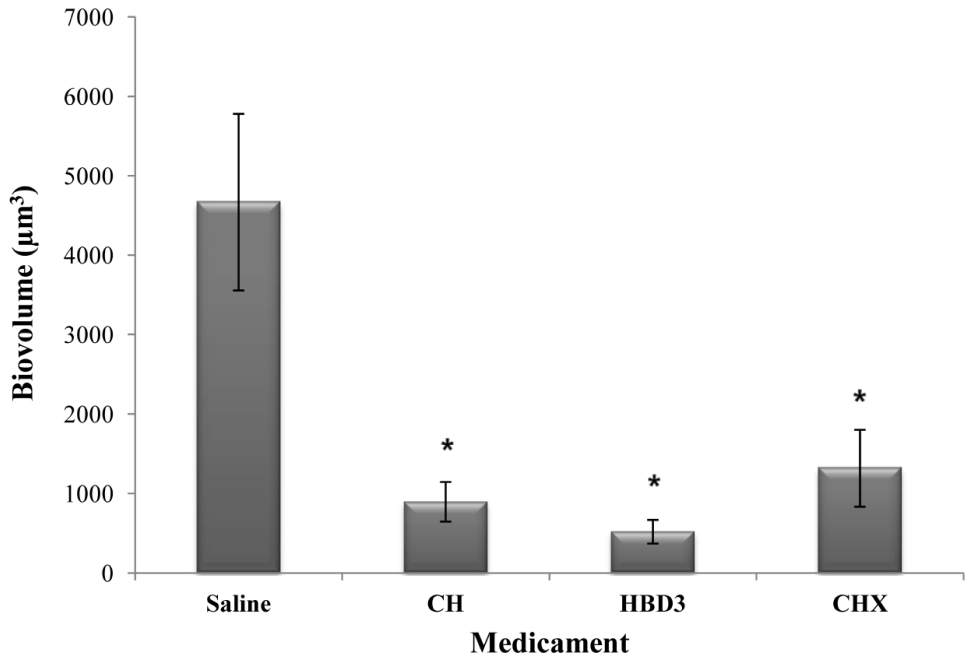

Figure 6. Effect of intracanal medicaments on the biovolume (μm^3) of multi-species biofilms. Biofilm samples composed of four bacterial strains were incubated for three weeks. The biofilms were treated for 24 hours with each medication and stained with commercial biofilm viability kit. The values of biovolume were calculated by the software bioImage_L (http://bioimagel.com).

CH, calcium hydroxide; CHX, 2% chlorhexidine digluconate solution; HBD3, human β-defensin-3 (50 µg/mL) solution. * denotes the significant difference between saline group and the other three medicaments. ($P < 0.001$)

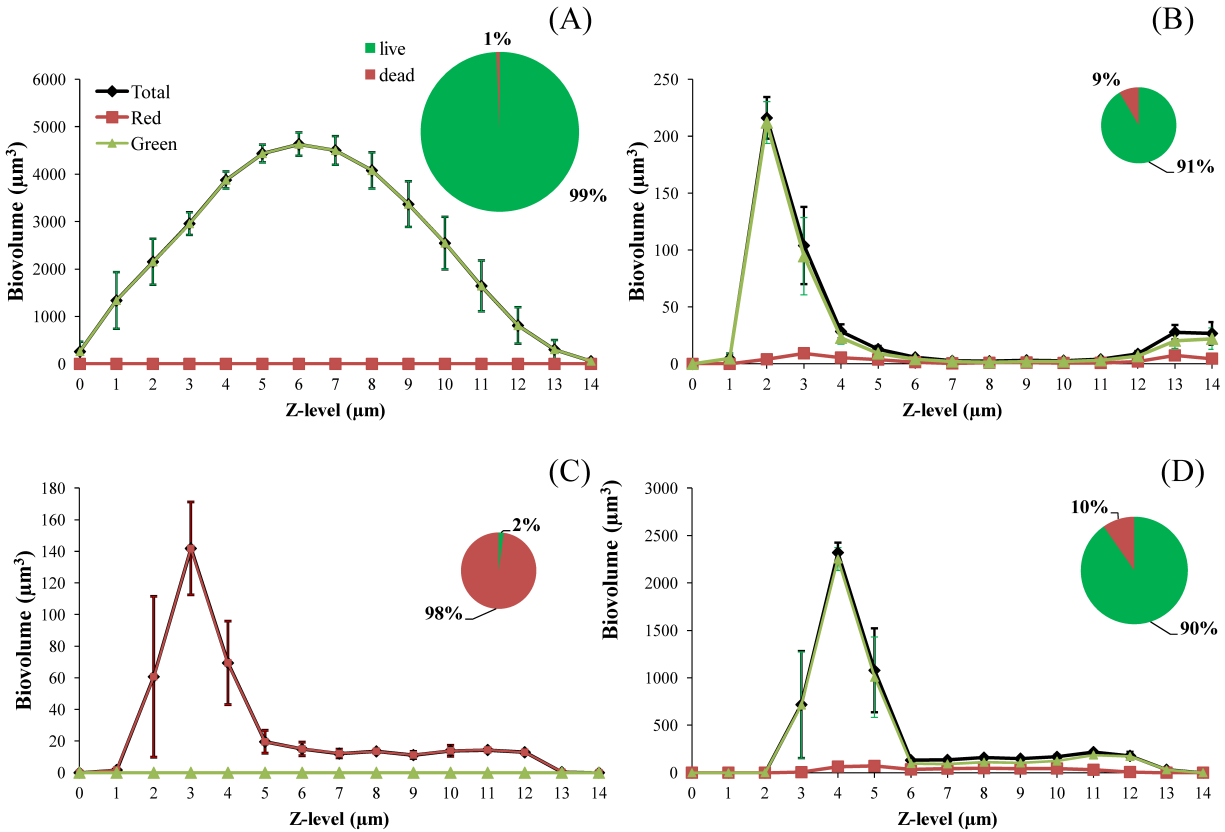

Figure 7. Biomass values of the total population and the green and red subpopulation corresponding to different z levels; (A) saline, (B) CH, (C) HBD3, and (D) CHX.

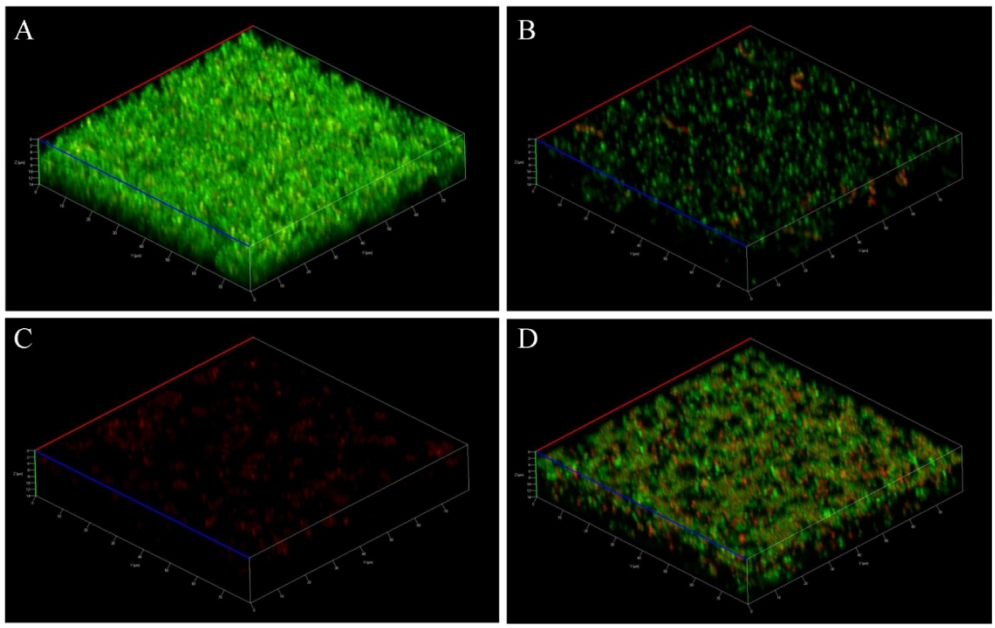

Figure 8. Three-dimensional construction of CLSM scans of biofilms stained with SYTO 9 and PI; (A) saline, (B) CH, (C) HBD3, and (D) CHX. Three randomly selected areas in each biofilm sample were imaged by CLSM. Fifteen-micrometer-deep scans (1-μm step size, 15 slices/scan) were obtained from each area. To visualize the profile of biofilms, all 15 slices/scan were reconstructed to three dimensional model by using the software ZEN 2010 (Carl Zeiss).

Figure 7. (continued from previous page) The bacterial cells were distributed throughout all z levels in the saline group, while most cells were located within 3 to 4 μm thickness in other medication groups. Any particular pattern depending on biofilm depth and medication was not observed. Error bars denote standard errors of triplicate experiments. The right pies of each graph show the distribution of dead cells (red) and live cells (green). The size of the circle is proportional to the volume of biofilms.

Table 1.The percentage of the dead cell volume of the entire volume in biofilms in the control and three experimental groups

saline	CH	HBD3	CHX
0.72 ± 0.17	8.51 ± 1.75*	96.8 ± 1.11*$^{+}$	9.75 ± 1.14*

CH, calcium hydroxide; HBD3, human β-defensin-3; CHX, chlorhexidine.

Values are mean ± S.D.

*Significant difference compared with Saline ($P < 0.05$)

+Significant difference compared with other medication group ($P < 0.05$)

국문초록

상아질 감염 모델 및 바이오필름 활성 검사를 이용한 human β-defensin-3 유래 펩타이드의 항균 효과 연구

이 진 경

서울대학교 학술대학원 치의학과 치과보존학 전공

(지도교수 금기연)

치수 및 치근단 조직의 염증질환은 여러 세균의 복합적인 감염에 의해 나타나며 특히 혼합된 세균들이 모여 근관 표면에 바이오필름을 형성하여 오래 지속되는 근관 감염을 야기할 수 있다. 본 연구에서는 *Enterococcus feacalis (E. faecalis)*로 감염된 상아질 감염 모델 및 바이오필름 활성 검사법을 이용하여, 주요 근관감염 세균으로 구성된 biofilm에 대한 Human beta-defensin-3 (HBD3)의 항균효과를 전통적인 근관소독제인 수산화칼슘 (CH) 및 chlorhexidine (CHX)의 항균능력과 비교하고자 하였다.

표준화된 상아질 블록에 *E. faecalis*를 감염시켜 3주간 배양하였다. 각 그룹당 12개의 블록 내부에 다음의 5가지 약제를 채웠다; CH를 물과 섞은 paste, 2% CHX 젤, HBD3 젤, 멸균식염수 (saline), 대조군 펩타이드 젤 (CP). 일주일 경과 후 LightSpeed file(Discus

Dental, Culver, USA)을 사용하여 근관 내부 200 및 400 μm 깊이 각각의 상아질 샘플을 얻었다. 72시간 배양 후 분광광도계를 이용하여 흡광도를 측정하였다. 결과 분석을 위해 반복 측정 변화 분석및 Tukey 사후 검정법을 이용하였다.

세균 활성 저하력을 보기 위한 균주로서 대표적 근관감염균인 *Actinomyces naeslundii, Lactobacillus salivarius, Streptococcus mutans*, 및 *E. faecalis*를 peptone-yeast-glucose 배지에 배양하여 동일한 비율로 섞어주었다. 이 혼합균을 조직배양접시에 넣은 멸균된 커버슬립에 접종한 후 3주간 배양하였다. 이 바이오필름 샘플에 saline, 포화된 CH 용액, 2% CHX 수용액, 50 μg/ml HBD3 수용액을 넣고 24시간 동안 처리하였다. 세균 활성 검사 키트를 사용하여 처리 후 공초점현미경 이미지를 얻어 죽은 세균의 퍼센트를 측정하였다. 일원배치 분산분석으로 통계 처리하였다.

상아질 블록 실험에서 HBD3 군은 200 및 400 μm 모두에서 CH 및 CHX 군보다 유의하게 낮은 흡광도 수치를 보였다 ($p < 0.05$). CH 군은 CP 및 saline 군과 유의한 차이가 없었다 ($p > 0.05$). 모든 그룹에서 200 μm와 400 μm 깊이에 따른 흡광도 수치의 차이는 없었다 ($p > 0.05$).

세균 생존 검사에서, 약제를 처리한 세 가지 군은 saline 군에 비해 생물체적(biovolume)에서 유의한 감소를 보였다 ($p < 0.05$). HBD3 군은 죽은 세포의 비율이 다른 약제군보다 현저히 높았다 ($p < 0.05$). CH 및 CHX 군은 멸균식염수 군보다 높은 항균 활성을 보였고 ($p < 0.05$), 두 그룹간 항균 활성에는 유의한 차이가 없었다 ($p > 0.05$).

결론적으로, HBD3는 상아질 블록 내 *E. faecalis* 및 주요 근관감염균으로 이루어진 바이오필름에 대해 CH 및 CHX보다 우수한 항균 효과를 보였다.

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주요어 : 항균성 실험, human β-defensin-3 peptide, 상아질 감염 모델, 흡광도, 바이오필름, 공초점 현미경.