치의학박사 학위논문

Inhibitory effect of a synthetic human β-defensin-3-C15 peptide on *Candida albicans* biofilm

Candida albicans 바이오필름에 대한 합성 human β-defensin-3-C15 peptide의 억제 효과

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Abstract

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Objectives.

The purpose of this study was to compare the inhibitory effect of a synthetic peptide comprising 15 amino acids of human β-defensin 3 (HBD3-C15) with chlorhexidine (CHX) or calcium hydroxide (CH) against *C. albicans* biofilm.

Methods.

To determine the minimal antifungal concentration (MAC), *C. albicans*

was grown on cover glass bottom dishes or human dentin disks for 48 h, and then treated with HBD3-C15 (0, 12.5, 25, 50, 100, 150, 200 and 300 μg/ml), CH (100 μg/ml) or Nys (20 μg/ml) for 7 days at 37℃. Using confocal laser scanning microscopy (CLSM) and field-emission scanning electron microscopy (FE-SEM), MAC was determined. To compare the inhibitory effect of HBD3-C15 peptide against *C. albicans* biofilms with conventional intracanal medicaments, *C. albicans* was also grown on cover glass bottom dishes or human dentin disks for 7 days, and then treated with HBD3-C15 (100 μg/ml, MAC), non-functional peptide (NP, 100 μg/ml), CH (100 μg/ml), 1% CHX, or saline for 7 days at 37 ℃. On cover glass, live and dead cells in the biomass were measured by the FilmTracer™ Biofilm viability assay, and observed by CLSM. On dentin disk, normal, diminished, or ruptured cells were observed by FE-SEM. The results were subjected to two-tailed *t*-test, one-way analysis variance and *post hoc* test at a significance level of P=0.05.

Results.

C. albicans survival on dentin was inhibited by HBD3-C15 in a dose-dependent manner. There were fewer aggregations of *C. albicans* in the groups of Nys and HBD3-C15 (≥100 μg/ml). CLSM showed C. *albicans* survival was reduced by HBD3-C15 in a dose dependent manner. Nys and HBD3-C15 (≥100 μg /ml) showed significant fungicidal activity compared to CH group ($P < .05$).

CLSM showed C. *albicans* survival was reduced by HBD3-C15 in a dose dependent manner. FE-SEM demonstrated that the *C. albicans* aggregated in HBD3-C15 (25 μg/ml) treated group and was disrupted in more than 100 μg/ml concentration of HBD3-C15 (MAC).

Live/Dead Biofilm viability assay and CLSM demonstrated that HBD3-C15 treated biofilms had a significantly less biovolume than CH, NP, and saline ($P < .05$), but had no significant difference from the CHX-treated group ($P > .05$). FE-SEM demonstrated that there was a marked decrease in aggregations of cells and biofilm and wrinkled or ruptured cells were frequently observed in the groups of CHX and HBD3-C15.

Conclusions.

Synthetic HBD3-C15 peptide (100 μg/ml) exhibited significantly higher antifungal activity than CH against *C. albicans* by inhibiting cell survival and biofilm growth, but had no significant difference compared to CHX.

Key words: *C. albicans* biofilm, confocal laser scanning microscopy, dentin disk, human β-defensin-3-C15 peptide, LIVE/DEAD Biofilm viability assay

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Contents

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Ⅰ. Introduction

Yeasts are typically opportunistic pathogens in the human oral cavity and have been detected in 7-18% of infected root canals (1). One of the predominant fungal pathogens in oral and vaginal infections is *Candida albicans* (*C. albicans*) (2). *Candida*, belongs to commensal microbes, often colonize the oropharyngeal, gastrointestinal, and vaginal microflora of

healthy patients (3). It invades dentinal tubules, colonize dentina l walls, and is considered to be dentinophilic microorganism (4,5).

Furthermore, *C. albicans* frequently forms biofilm (5,6) that is more resistant to antifungal agents such as fluconazole (6,7). The resistance of the biofilms is due to nutrient limitation and slow growth, poor antibiotic penetration, adaptive stress responses, and formation of persister cells (7,8). Actually, *Candida* contamination of the root canals could be closely associated with endodontic treatment failure (4, 9, 10).

Microbial infection is the main cause of apical periodontitis (11). Nonetheless, contemporary instrumentation techniques or instrument types cannot completely eliminate microorganisms from infected root canal systems (12, 13). The remaining microorganisms grow and multiply within the root canals unless antimicrobial medicaments are additionally used between appointments (14). Therefore, intracanal medicaments have been used to disinfect the root canals and eradicate the remaining microorganisms (15). However, calcium hydroxide (CH), the most commonly used medicament for root canal disinfection, has been reported to be ineffective against *C. albicans* (16, 17). This may be due to the low solubility and diffusibility of CH (18), or the resilience of *C. albicans* in alkaline environment (19). Nystatin (Nys) is one of the efficient antifungal agents

against *C. albicans* (20). Nonetheless, the antifungal resistance of Nys increased when the drug was repeatedly used (21).

Although chlorhexidine (CHX) has shown antifungal effects against *C. albicans* (19), phenotypic resistance was exhibited by subpopulations of cells within biofilms (22). Furthermore, antifungal antibiotics used to treat fungal infection, have shown risks of developing resistance and host sensitization from repeated use (14). Thus, a search for novel intracanal medicaments including innate antifungal peptides is required (23).

Antimicrobial peptides (AMPs) found in the oral cavity serve as host defense (24). These include human β-defensins (HBDs), which are positively charged, cysteine-rich peptides expressed in inflamed pulps (25). The predicated HBD sequence is available over forty HBDs, the native peptide has been isolated for three HBDs (HBD1, 2, and 3) (24). The HBD1, 2, and 3 participate innate immune response, and the HBD3 was found to have the strongest antimicrobial activity (26). Since natural HBD3 is 67 amino acids long and unstable (27), synthetic peptide fragments of HBD3 were suggested as antibacterial agents (24). The HBD3 possessed a broad spectrum of antimicrobial activities against Gram-negative bacteria, Gram-positive bacteria and fungi with strong immunoregulatory activity (28).

Synthetic HBD3 derivatives have been reported to have significant

antimicrobial activity that is comparable to the natural antimicrobial peptides (29). For example, a synthetic HBD3-C22 peptide with C-terminal 22-mer was reported to have antifungal activity against *C. albicans* (30).

The synthetic HBD3 peptide with C-terminal 15-mer (HBD3-C15, NIBEC, Seoul, South Korea) has antimicrobial efficacy against both *E. faecalis* biofilms (31) and multispecies biofilms (32), but its antifungal activity is unknown. Therefore, the purpose of this study was to compare the inhibitory effect of a synthetic HBD3-C15 peptide with CH and CHX against *C. albicans* biofilm.

Ⅱ. Materials and Methods

Minimum inhibitory concentration (MIC) and minimum antifungal concentration (MAC) of HBD3-C15 against planktonic C. albicans

To access the inhibitory effect of HBD3-C15 against planktonic *C. albicans,* the MIC and the MFC were determined by using the microdilution methods (33). *C. albicans* at 1×10^7 colony forming units (CFU) in mid-log phase was inoculated into 96-well polystyrene plates (Thermo, Waltham, MA, USA) by 50 μl with the equal volume of HBD3-C15 at 0, 1.58, 3.17, 6.25, 12.5, 25, 50, and 100 μg/ml at 37°C for 3 h. Then, the mixture was

inoculated on yeast malt (YM) agar plate and live *C. albicans* was enumerated by CFU at the following day. The experiments were performed three times. Through this procedure, the MIC and MFC of HBD3-C15 against planktonic *C. albicans* biofilm was determined (Fig. 1).

Determination of inhibitory concentration of HBD3-C15 on C. albicans biofilm

This study was approved by the Institutional Review Board (IRB) of Seoul National University Dental Hospital(CRI 15007). Single-rooted premolars with fully formed apices (N=8) were collected from patients undergoing extractions for orthodontics in the Department of Oral and Maxillofacial Surgery at Seoul National University Dental Hospital. Calculus and soft tissue on the root surfaces were removed by an ultrasonic scaler, and the teeth were stored in sodium azide (0.5%, Sigma-Aldrich, St. Louis, MO, USA) at 4 °C. The roots were sliced into 500 μm-thick cross sections (Fig. 2) using an Isomet precision saw (Buehler, Lake Bluff, IL, USA). These dentin disks were treated with 17% ethylenediaminetetraacetic acid (EDTA, pH 7.2, Sigma-Aldrich) for 5 min, followed by sodium hypochlorite (2.5%, Sigma-Aldrich) for 5 min, then neutralized with 5% sodium thiosulfate (Sigma-Aldrich) for 5 min, and finally washed three times with distilled

water. They were then autoclaved for 15 min at 121 °C and incubated in liquid growth medium containing peptone-yeast-glucose in 10 mmol/l potassium phosphate-buffered saline (pH 7.5) at 37 °C for 24 h to ensure sterility. *C. albicans* (KCTC 7270, Korea Collection for Type Cultures, Daejeon, Korea) was grown in yeast malt media at 37 °C until the microbe reached mid-log phase (A_{600}=0.1).

To determine the minimal antifungal concentration (MAC) of HBD3-C15 against *C. albicans* biofilm, five dentin disks were incubated with cell aliquots (300 μl/well, 6×10^6 cells/ml) in 24-well plates for 48 h, and then treated with synthetic HBD3-C15 (0, 12.5, 25, 50, 100, 150, 200 and 300 μg/ml, NIBEC), CH (100 μg/ml) or Nys (20 μg/ml) for 7 days at 37 °C. Also three thousand microliters of *C. albicans* suspension was transferred to each well of the cover glass bottom dish (SPL, Seoul) for biofilm formation for 48 h. The biofilm samples was treated with HBD3-C15 (0, 12.5, 25, 50, 100, 150, 200 and 300 μg/ml), CH (100 μg/ml) or Nys (20 μg/ml) for 7 days at 37 °C. After 1 week medication, the dentin disks were thoroughly washed out using PBS and then examined by field-emission scanning electron microscopy (FE-SEM; S-4700, Hitachi, Tokyo, Japan, 1,000x, 5,000x, 30,000x). On cover glass, live and dead cells in the biomass were measured by the FilmTracer Biofilm viability assay, and observed by

confocal laser scanning microscopy (CLSM, LSM 5 pascal, Carl Zeiss, Jena, Germany). Through this procedure, the MAC of HBD3-C15 against *C. albicans* biofilm was determined.

FE-SEM observation for antifungal comparison

To compare the inhibitory effect of HBD3-C15 with conventional intracanal medicaments such as CH or CHX, *C. albicans* was grown on human dentin disks for 1 week, and then treated with and HBD3-C15 (100 μg/ml, MAC), non-functional peptide (NP, 100 μg/ml), CH (100 μg/ml), 1% CHX, or saline for 1 week at 37 °C. The HBD3-C15 and NP were prepared by F-moc-base chemical solid-phase (30). In NP the C-terminal fifth and sixth L-cysteines of HBD3-C15 were substituted with alanine. After 1 week medication the five of the dentin samples were prepared for FE-SEM (1,000x, 5,000x, 30,000x). On dentin, normal, diminished and ruptured cells were observed by FE-SEM.

LIVE/DEAD Biofilm viability assay for antifungal comparison

C. albicans mid-log phase cultures (3 ml/dish, 6×10^6 cells/ml) were transferred to a cover glass bottom dish (SPL) and incubated for 7 days, and then treated with HBD3-C15 (100 μg/ml), saturated CH (100 μg/ml) , 1%

CHX, NP (100 μg/ml), or 0.9% sterile saline for 7 days at 37 °C. Each sample was washed gently with PBS. The FilmTracer LIVE/DEAD Biofilm viability kit (Molecular Probes, Carlsbad, CA, USA) containing SYTO9 and propidium iodide (PI) was used to stain live and dead *C. albicans* in the biofilms. The stained *C. albicans* biofilm was viewed using CLSM (LSM 700) with the ×40 lens. SYTO 9 stains both live and dead microorganisms in fluorescent green, whereas PI only stains the nucleic acids of cells with damaged membranes and thereby identifies dead microbes (32). The stained *C. albicans* biofilms were examined by CLSM (LSM 700, Carl Zeiss) with the ×40 lens. CLSM images were acquired by using ZEN 2010 (Carl Zeiss) software at a resolution of 512 × 512 pixels with a zoom factor of 2.0. Each 2-dimensional (2D) image covered an area of 230.34 × 230.34 μm. The three-dimensional (3D) reconstructed images had a z step of 1 μm in each stack, and there were 15 stacks in total. The percentage of dead cells was determined from the ratio of biovolumes for the red subpopulation to that of red or green population.

Image and statistical analyses

The CLSM images were analyzed with bioImage_L(http://bioimagel.com) software. The green and red stained portions of the biofilm were used to

calculate live and dead cell subpopulations within the total biomass. Statistical significance was examined using a two-tailed *t*-test, a one-way analysis of variance and a *post hoc* test at a significance level of P=0.05, using SPSS ver 22 (SPSS Inc., Chicago, IL, USA).

Ⅲ. Results

Assessment of antifungal concentration of HBD3-C15

The MIC and MAC of HBD3-C15 against planktonic *C. albicans* were 3.175 (1.6×10^4 CFU/ml) and 50 μg/ml, respectively. HBD3-C15 peptide showed complete inhibition of planktonic *C. albicans* at 50 μg/ml (Fig. 1).

HBD3-C15 peptide and Nys inhibited the growth of C. albicans biofilm on human dentin

The colonization of *C. albicans* for biofilm formation on dentin disks was inhibited by HBD3-C15 in a dose-dependent manner (Fig. 3). Comparing with CH group, there were fewer *C. albicans* aggregated on dentin in the groups of HBD3-C15 (≥100 μg/ml) and Nys (Fig. 3) under FE-SEM. Ruptured cells were also observed at the concentrations of 150 to 300 μg/ml of HBD3-C15 and Nys (Fig. 3).

HBD3-C15 and Nys reduced C. albicans survival and biofilm

CLSM demonstrated that *C. albicans* survival and biofilm were reduced by HBD3-C15 in a dose-dependent manner (Figs. 4A and 4B). The dead cell biomass in the HBD3-C15 (≥100 μg/ml) and Nys was significantly more ($P < 0.05$) than that of CH group (Fig. 4C), but not significantly ($P < 0.05$) different between HBD3-C15 (300 μg/ml) and Nys. Finally, 100 μg /ml of HBD3-C15 peptide was used as the MAC against *C. albicans* biofilm.

FE-SEM observation of inhibitory effect of tested medicaments against 1 week -old biofilm

When 1 week old biofilms of *C. albicans* were treated with medicaments for 1 week, there was a marked decrease in aggregations of cells and biofilm in the CHX and HBD3-C15 groups (Fig. 6). There was intact biofilm in the NP and saline groups, reduced cells in the CH group, some cells in the CHX group, and very few cells in the HBD3-C15 group. Additionally, wrinkled and ruptured cells were frequently observed with HBD3-C15 treatment. The HBD3-C15 appeared to disrupt *C. albicans* membranes and to markedly inhibit their aggregation.

Live/Dead Biofilm viability assay and CLSM observation of medicated C. albicans biofilms

The biofilms that had been treated with saturated CH, 1% CHX or HBD3-C15 (MAC) had less proportion of live cells and higher proportion of dead cells than the NP or saline groups ($P < 0.05$, Figs. 7A). HBD3-C15 treated biofilms had a significantly less biovolume than CH, NP, and saline ($P < 0.05$), but had no significant difference from the CHX-treated group ($P > 0.05$, Fig. 7B).

Dimensional analysis of C. albicans biofilms

The biomass for the total population of fungal cells appeared to be normally distributed across the z level plot (0-15 μm). Their highest densities were at around 6 μm in the CH, NP and saline (Figs. 8A, 8B and 8C), and at around 7-8 μm in the CHX and HBD3-C15 treated biofilms (Figs. 8D and 8E). Dead (red) cells increased in the HBD3-C15 treated biofilms.

Ⅳ. Discussion

This study compared the inhibitory effect of synthetic HBD3-C15

peptide against *C. albicans* biofilms with CH and CHX, and showed HBD3-C15 has better inhibitory effect than CH and slightly better than CHX. Song et al. demonstrate that recombinant HBD3 protein was shown to be bactericidal against endodontic pathogens (34). However, the whole HBD3 has a long amino acid sequence which is harder to synthesize, and its antibacterial/antifungal activity is attenuated at elevated ionic strength (35). Rather, the shorter HBD3-C15 peptide might be anticipated to have the potential for greater antibacterial/antifungal activity at a reduced cost.

Defensins are short and positively charged peptides marked by the presence of conserved cysteines (36). Cysteine residues serve to chemotaxis activities and their overall peptide structural stability (35), and also contribute to the antibacterial activity of the HBD3 (25). NP peptide of which the C-terminal cysteine was replaced with alanine, did not show superior antifungal activity compared to HBD3-C15. Similar effects are also found in other AMPs, such as tachyplesin and polyphemusins (35).

HBD3-C15 binds to negatively charged cell membranes by electrostatic interactions (37), disrupts their integrity and causes leakage of cellular contents and cell destruction(38). Similarly, CHX is a cationic bisbiguanide that collapses the membrane potential at inhibitory concentration (0.2%), causing membrane disruption (39) and leakage of

intracellular components (40). Compared to CHX, HBD3 not only inhibits biofilm formation but also reduces the preexisting biofilm by inhibiting polysaccharide synthesis at the level of gene transcription (41).

Nystatin is polyene antifungal antibiotics and its fungicidal effect by an alteration of cellular permeability causes loss of essential components from the cell (42), thereby leading to the cell death (43). Nystatin (20 μg/ml) showed comparable effect to HBD3-C15 (200 μg/ml) against *C. albicans* biofilm in the present study. Nonetheless, the administration of the antibiotic into the root canal system is with the potential risk of adverse systemic effects, particularly toxicity, allergic reactions, and development of resistant strains of microbes (44).

The antifungal mechanism of HBD3 against *C. albicans* involves specific interactions with the cell-surface proteins Ssa (45), causing cell membrane disruptions (46). In the present study, ruptured *C. albicans* cells were frequently observed in the high concentrations of HBD3-C15. This suggests that HBD3-C15 might increase the permeability of fungal cell membranes, thereby enhancing their uptake of PI, which is a small molecule (668.39 Da) that cannot cross intact membranes. However, the details of this killing mechanism are not still understood. Further studies will be needed to

fully elucidate the antifungal pathways of HBD3-C15 peptide at the molecular level.

FE-SEM showed the disruption and few aggregations of *C. albicans* cells in the HBD3-C15 and CHX-treated groups. Furthermore, CLSM evaluation showed that the proportion of dead microbes in the HBD3-C15 and CHX-treated biofilms were significantly higher than that of the CH. Ballal et al. demonstrated that the antifungal activity of CHX was more effective than CH against *C. albicans* (47), which was consistent with the present study. This might be due to the bacterial substantivity of CHX, which might prevent the *Candida* colonization as it prevented the bacterial colonization on dentinal surface (38).

Regarding the antifungal effect of CH, it had limited efficacy in killing *C. albicans* biofilm in our study, which is consistent with the previous report (48). *C. albicans* could survive at a wide range of pH and the alkalinity of aqueous CH may not have antifungal effect on *C. albicans* (10, 12). The antifungal efficacy of the CH was high in the first 24 h against *C. albicans*, whereas the efficacy gradually reduced after 72 h. This might be attributed to the dilution of the CH as time progressed (37) or dentin buffering effect (40). In addition, CH may provide the Ca^{2+} ions necessary for the growth and morphogenesis of *Candida* (49). These mechanisms may

explain why CH has been found to be ineffective against *C. albicans* (10, 12).

Collectively, the present results suggest that the HBD3-C15 peptide may be effective as an intracanal antifungal medicament. HBD3 had a very low cytotoxicity against host cells (37). Its antimicrobial efficacy and range of target microorganisms could be modulated by designing analogs of HBD3 (28). Compared with CH or CHX, the HBD3-C15 peptide gel provided greater antibacterial effects against *E. faecalis* (31) biofilm and multispecies biofilms (32). Furthermore, the HBD3-C15 peptide gel has a low viscosity that will prolong its contact with dentinal tubules and root canal walls, and promote its delivery to inaccessible isthmuses, canal fins, and curved canals. Considering the present anti-fungal effect with the antimicrobial effects against endodontic fungal pathogens (25, 34), the HBD3-C15 peptide could be applied as an injectable intracanal medicament for therapy-resistant/persistent root canal infection or for endodontic regenerative procedure of infected immature permanent tooth. Further study is planned using dentin infection model as substrate to mimic clinical root canal environments.

Ⅴ. Conclusions

Synthetic HBD3-C15 peptide exhibited higher inhibitory activity than CH by inhibiting cell survival and biofilm growth against *C. albicans*, but had no significant difference compared to CHX.

VI. References

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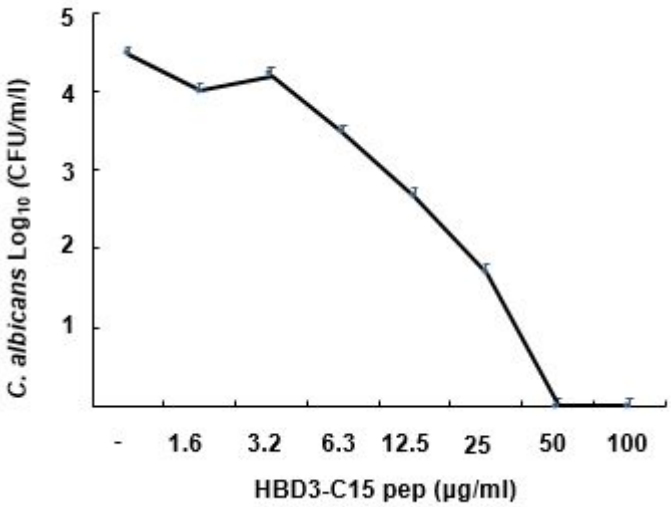


Figure 1. The MIC and MFC of HBD3-C15 against planktonic *C. albicans* were 3.175 and 50 μg/ml, respectively. *C. albicans* (KCTC7270) cultured in yeast malt (YM) broth to midlog phase was inoculated into a 96-well polystyrene plate and incubated with the equal volume of HBD3-C15 at 0, 1.588, 3.175, 6.25, 12.5, 25, 50, and 100 μg/ml at 37°C for 3 h. Then, the mixture was inoculated on YM agar plate and live *C. albicans* was enumerated by counting colony-forming unit (CFU).

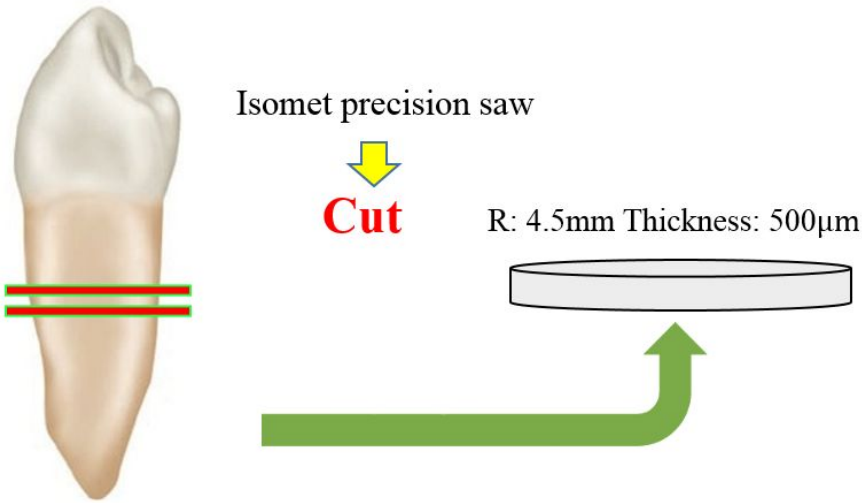


Figure 2. Schematic draw of construction of dentin disk using a microtome (Isomet precision saw). Calculus and soft tissue on the root surfaces were removed by an ultrasonic scaler. The roots were sliced into 500 μm-thick cross sections.

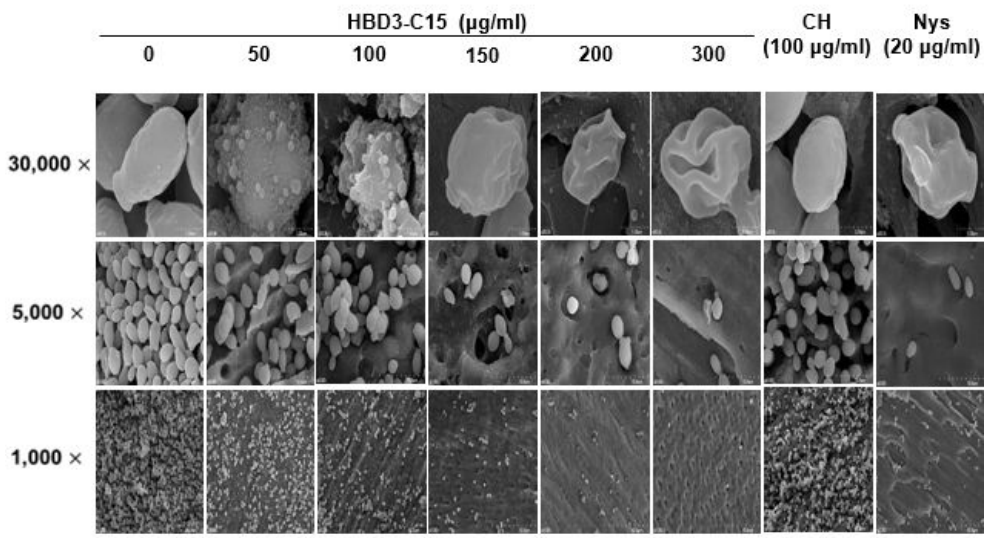


Figure 3. Field-emission scanning electron microscopic images of HBD3-C15, calcium hydroxide, and Nystatin treated dentinal disks. *C. albicans* (6×10^6 cells/ml) was incubated for 48 h and treated with either HBD3-C15 (0, 12.5, 25, 50, 100, 150, 200 or 300 μg/ml), aqueous calcium hydroxide (CH, 100 μg/ml), and Nystatin (20 μg/ml) for 7 days. Morphological changes of *C. albicans* were observed using field-emission scanning electron microscope.

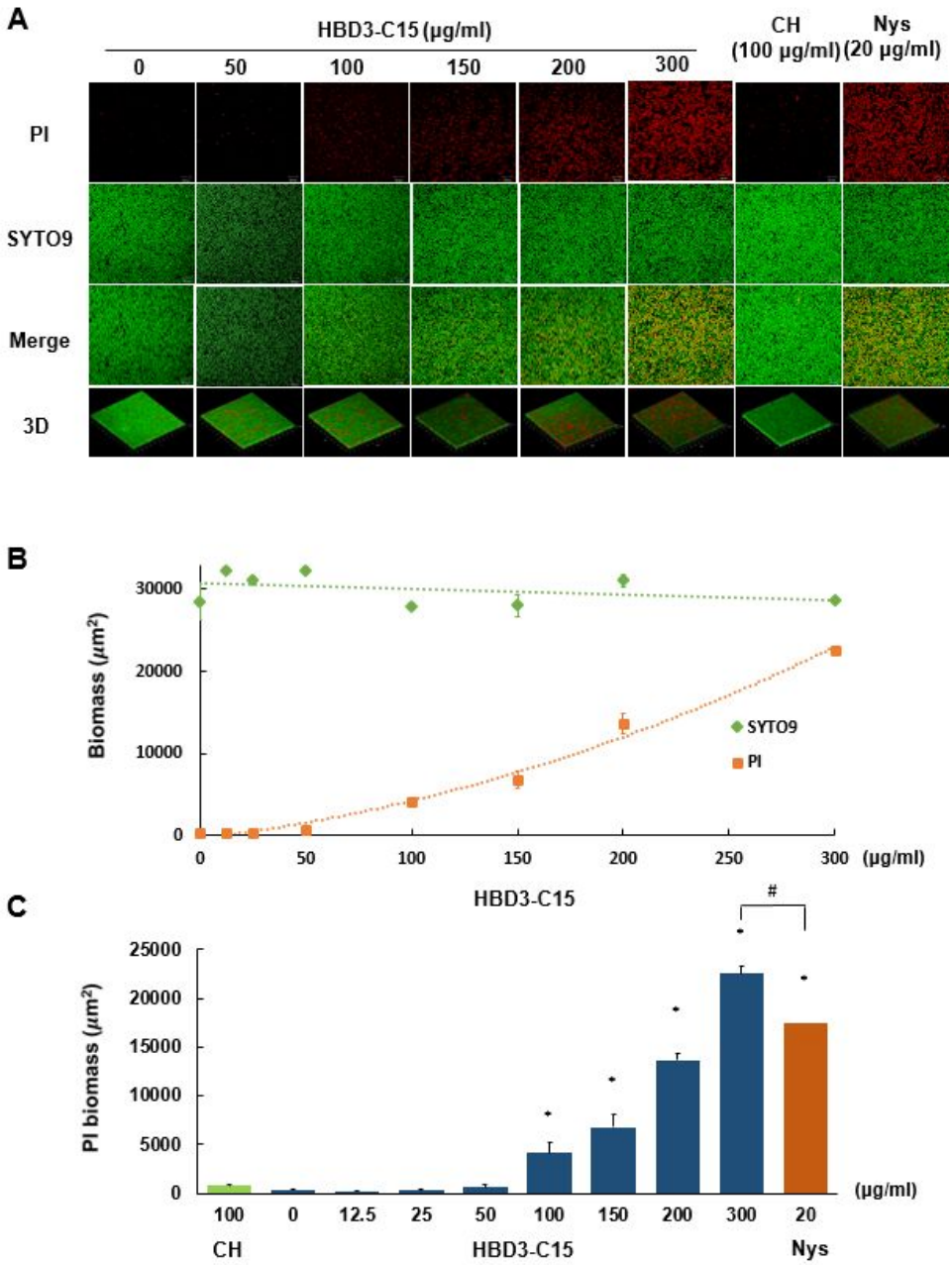

A
HBD3-C15 (μg/ml)
0
50
100
150
200
300
CH (100 μg/ml)
Nys (20 μg/ml)
PI
SYTO9
Merge
3D
B
Biomass (μm²)
30000
20000
10000
0
SYTO9
PI
0
50
100
150
200
250
300
(μg/ml)
HBD3-C15
C
#
PI biomass (μm²)
25000
20000
15000
10000
5000
0
(μg/ml)
100
0
12.5
25
50
100
150
200
300
20
CH
HBD3-C15
Nys

Figure 4. LIVE/DEAD Biofilm Viability assay of tested medicaments against *C. albicans* biofilms. *C. albicans* (6×10^6 cells/ml) was incubated for 48 h on cover glass and treated with either HBD3-C15 (0, 12.5,25, 50, 100, 150, 200, 300 μg/ml), aqueous calcium hydroxide (CH, 100 μg/ml), and Nys (20 μg/ml) for 7 days. FilmTracer LIVE/DEAD Biofilm Viability staining was performed and observed under CLSM (A). The numbers of dead cells (PI stained red) were increased in the group of HBD3-C15 in a dose-dependent manner (B). The dead cell biomass were significantly higher in the groups of HBD3-C15 (≥100 μg/ml) and Nys than CH group ($P < 0.05$, C).

# indicates no significant difference between HBD3-C15 (300 μg/ml) and Nys ($P < 0.05$).

*Significant difference compared with CH groups ($P < 0.05$).

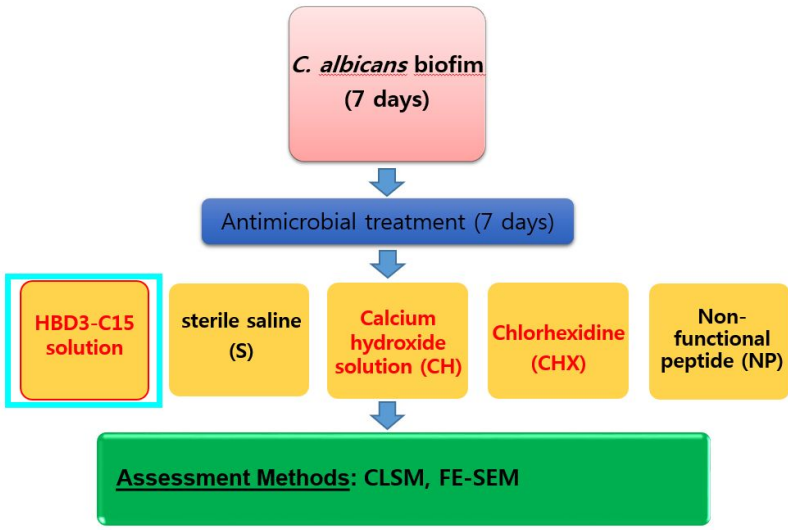


Figure 5. The experimental flow chart for comparing the inhibitory effect of HBD3-C15 with CH and CHX against 1 week-old *C. albicans* biofilm. (HBD3-C15: Human beta defensing 3 peptide with C-terminal 15-mer)

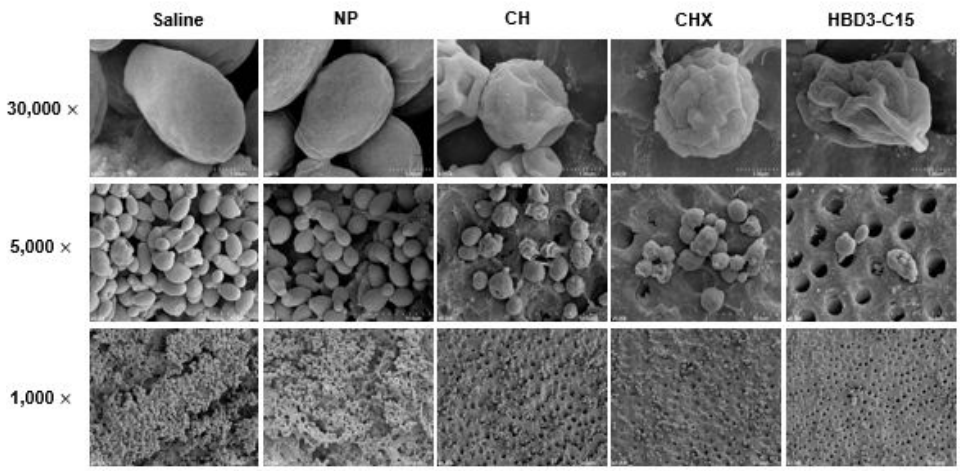
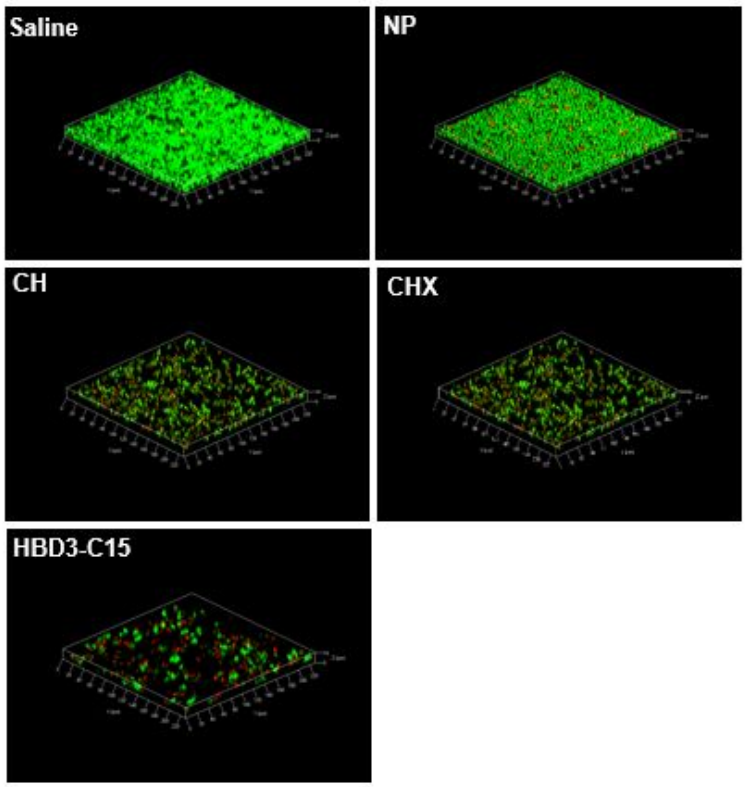


Figure 6. Field-emission scanning electron microscopic images of *C. albicans* biofilms on dentin. *C. albicans* (6×10^6 cells/ml) was incubated on dentin for 7 days and treated with either hBD3-C15 (100 μg/ml), nonfunctional peptide (NP, 100 μg/ml), calcium hydroxide (CH, 100 μg/ml), chlorhexidine (CHX, 1%), or saline for 7 days. Morphological changes of *C. albicans* cells were observed using field-emission scanning electron microscope. (1000×, 5000×). Cells that appeared normal (saline and NP) or wrinkled squashed walnut-shape (CH, CHX, and HBD3-C-15) were seen (30,000×).

A


Saline
NP
CH
CHX
HBD3-C15

B

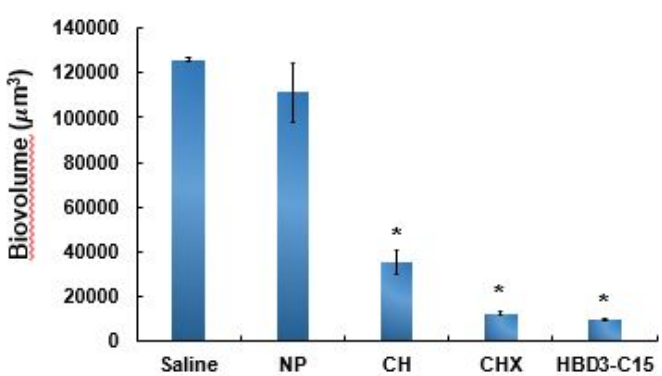

Biovolume (μm³)
140000
120000
100000
80000
60000
40000
20000
0
*
*
*
Saline
NP
CH
CHX
HBD3-C15

Figure 7. Biovolumes (μm^3) of *C. albicans* biofilms on cover glass. *C. albicans* (6×10^6 cells/ml) was incubated on cover glass for 7 days and treated with either HBD3-C15 (100 μg/ml), nonfunctional peptide (NP, 100 μg/ml), calcium hydroxide (CH, 100 μg/ml), chlorhexidine (CHX, 1%), or saline for 7 days. (A) FilmTracer LIVE/DEAD Biofilm Viability staining and examination by CLSM followed by 3-dimensional reconstructions, showed fewer live (green) cells and some dead (red) cells with CH, CHX, and HBD3-C15. (B) BioImage_L software calculations showed significantly less biovolume in the CHX and HBD3-C15.

* and ¥ indicates no significant differences between inter-group ($P > 0.05$)

# indicates significant difference ($P < 0.05$).

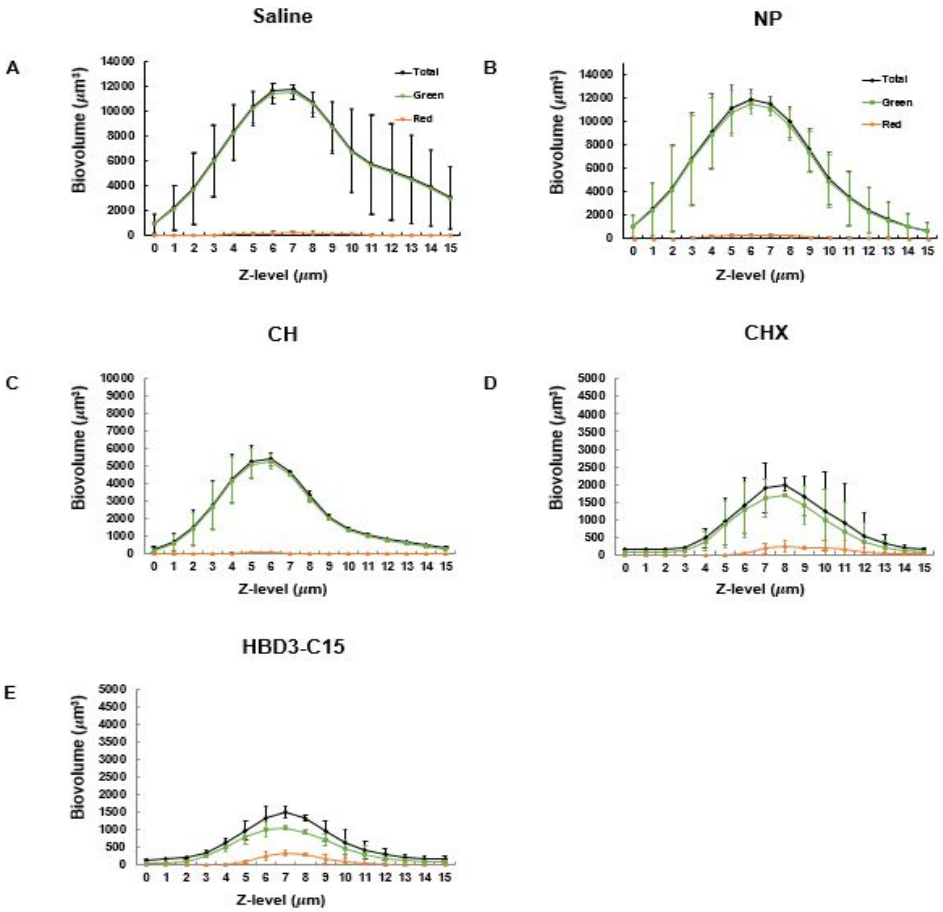


Figure 8. Biomass values of total population and green and red subpopulation corresponding to different z levels: (A) saline, (B) non-functional peptides (100 μg/ml), (C) CH (100 μg/ml), (D) 1% CHX, (E) HBD3-C15 (100 μg/ml). The *C. albicans* cells distributed throughout z levels. Any particular pattern depending on biofilm depth and medication was not observed.

국문초록

Candida albicans biofilm 에 대한 합성 human β-defensin-3-C15 peptide 의 억제효과

치과보존학 전공 임 상 민

(지도교수 금 기 연)

목 적

본 연구에서는 *Candida albicans* 바이오 필름에 대한 15개 아미노산을 함유하는 합성 펩타이드 (HBD3-C15) 의 성장 억제효과를 calcium hydroxide (CH) 및 chlorhexidine (CHX) 와 비교평가 하였다.

방 법

합성 HBD3-C15 peptide의 최소 항진균 농도를 결정하기 위하여 *C. albicans* 를 사람 상아질 디스크와 커버글라스에서 48시간 동안 배양하여 biofilm을 형성시키고, 각각 0, 12.5, 25, 50, 100, 200, 300

μg/ml HBD3-C15, CH (100 μg/ml), Nys (20 μg/ml) 로 처리하여 7일 동안 37℃에서 배양하였다. 이를 공초점 현미경과 field emission scanning electron microscopy (FE-SEM)을 사용하여 최소 항진균 농도를 결정하였다. 결정된 HBD3-C15 의 최소 항진균 농도와 기존에 사용되어 온 다른 약제와 진균 억제 효과를 비교하기 위하여, *C. albicans* 를 사람 상아질 디스크와 커버글라스에서 7일 동안 배양하여 biofilm을 형성시키고, 각각 100 μg/ml HBD3-C15, non-functional peptide (NP), saturated CH, 1% CHX, saline 으로 처리하여 7일간 37 ℃에서 배양하였다. 커버글라스에 형성된 biofilm은 세균활성검사 키트를 사용하여 처리 후 공초점 현미경 이미지를 얻어 살아있는 세포와 죽은 세포를 측정하였다. 상아질에서는 정상, 크기가 작아진, 파열된 세포를 FE-SEM으로 관찰하였다. 결과분석을 위하여 two-tailed t-test와 일원배치 분산분석을 시행하였다 ($P < 0.05$).

결 과

공초점 현미경 분석에서 *C. albicans* 는 HBD3-C15 농도에 비례하여 억제되는 양상을 보였다. FE-SEM 분석결과에서도 상아질 표면에서

C. albicans 생존이 HBD3-C15 농도가 증가할수록 감소하였다. 낮은 농도의 HBD3-C15 에서는 *C. albicans* 집락이 관찰되었고, 높은 농도 (100 μg/ml 이상) 에서는 세포막이 파열된 세포가 관찰되었다. 다른 약제와의 진균억제 효과 비교 실험에서는, 100 μg/ml 로 HBD3-C15 로 처리된 바이오 필름은 공초첨 현미경 상에서 CH, NP, saline과 비교하여 통계적으로 유의하게 바이오 볼륨이 더 낮았으며 ($P < 0.05$), CHX와 비교해서는 통계적으로 유의한 차이가 없었다 ($P < 0.05$). FE-SEM 분석 결과에서 CHX과 HBD3-C15에서 눈에 띄는 세포 군집과 바이오 필름의 감소가 관찰되었고 주름지고 파괴된 세포가 더 자주 관찰되었다.

결론

합성 HBD3-C15 peptide (100 μg/ml) 는 *C. albicans* 세포의 생존과 biofilm 성장을 억제함으로써 CH 에 비하여 더 높은 항진균 효과를 보였고, CHX 와 비교해서는 유의한 차이를 보이지 않았다.

주요어: *C. albicans* biofilm, 공초점 현미경, 상아질 디스크, Human ß-defensin3-C15 peptide, 세균활성검사키트

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