Chiang Mai Dental Journal

Original Article

Antibiofilm Effect of Citric Acid-modified Chlorhexidine Gluconate on a Dual-species Biofilm

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Received: June 21, 2021 • Revised: July 22, 2021 • Accepted: August 4, 2021

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Abstract

Objective: The purpose of this study was to evaluate the antibiofilm effect of chlorhexidine gluconate (CHX) added with citric acid (CA) on bacterial-fungal biofilm.

Methods: Dentin slices were sectioned from the crown of extracted human third molars. After sterilization, samples were inoculated with Enterococcus faecalis *(E. faecalis)* and Candida albicans *(C. albicans)* for 14 days to establish a bacterial-fungal biofilm. All samples were randomly divided into four treatment groups: phosphate-buffered saline (PBS) (negative control), 2% CHX, 10% CA, and 10% citric acid-modified 2% chlorhexidine gluconate (CAmCHX). Each dentin slice was treated with one of the selected solutions for 1 minute. Following treatment, samples were labeled with a fluorescent viability stain to identify live and dead cells. The proportion of dead cells to total cells was analyzed with confocal laser scanning microscopy (CLSM), and biofilm removal efficacy was evaluated by scanning electron microscopy (SEM).

Results: 10% CA and CAmCHX groups demonstrated a higher proportion of dead cells to total cells than the PBS group ($p<0.05$). No significant difference was observed between the 10% CA and CAmCHX groups ($p>0.05$). SEM images revealed less remaining biofilm in 10% CA and CAmCHX groups. Whereas, in 2% CHX and PBS groups, the biofilm structure was still intact.

Conclusions: Within the limitations of this study, 10% CA and CAmCHX demonstrated an antibiofilm effect against *E. faecalis* and *C. albicans* biofilm on the surface of dentin slices. CAmCHX can be thought as an alternative choice for irrigation to remove the biofilm. Future study should focus on the cytotoxicity of this agent prior to clinical used.

Keywords: biofilms, *Candida albicans*, chlorhexidine, citric acid, *Enterococcus faecalis*

Introduction

Microorganisms are the cause of endodontic infection.[1] The goal of root canal treatment is to eradicate and prevent the recontamination of microorganisms. These microorganisms grow as both planktonic and biofilm. A biofilm is a group of biodiverse organisms encapsulated with extracellular polymeric substances (EPS), providing protection from the threatening environment.[2] *Enterococcus faecalis (E. faecalis)* and *Candida albicans (C. albicans)* are the most common bacteria and fungi, respectively, found in root canal infection with post-treatment disease.[3]

E. faecalis is a gram-positive, facultative anaerobic bacteria. This bacteria can survive in root canal-treated teeth for 12 months.[4] It was hypothesized that *E. faecalis* have a stress-response mechanism by changing into the viable but non-culturable (VBNC) state.[5] This is a response to the unfavorable environment for growth, such as low

nutrient, high pH, and osmolarity imbalance. *E. faecalis* in the VBNC state will not grow in culture media. However, their viability and virulence are still retained. *C. albicans* is a pleomorphic fungus that enters the root canal system through contamination during or after root canal treatment.[6] Both microbes can withstand a highly alkaline environment, resulting in a decreased effectiveness of many root canal medicaments, including calcium hydroxide. There are interactions between *E. faecalis* and *C. albicans* in biofilms with *C. albicans* prolonging the infection of *E. faecalis,* which ultimately leads to persistent infection.[7]

The American Association of Endodontists[8] recommends the use of ethylenediaminetetraacetic acid (EDTA) followed by sodium hypochlorite (NaOCl) as final flush irrigants. EDTA removes the smear layer, whereas NaOCl removes microorganisms along with the residual biofilm. The protocol involves multi-step procedures, moreover, NaOCl and EDTA lack substantivity.[9] A study also indicated that EDTA causes depletion of calcium and erosion of root dentin, which affects tooth restoration processes.[10] There were also concerns about the toxicity of both NaOCl and EDTA.[11]

Citric acid (CA) has the ability to remove the smear layer and biofilm.[12,13] An *in vitro* study reported an efficient smear layer removal capability of 10% CA.[14] Unlike chlorhexidine gluconate (CHX), which cannot remove the smear layer nor biofilm.[15] However, the antimicrobial property of CA is relatively limited.[16] Whereas CHX is a broad-spectrum antimicrobial agent with a pH of 5-7. The antimicrobial properties of CHX are either bactericidal or bacteriostatic depending on its concentration, and it is also effective against persistent endodontic microorganisms such as *E. faecalis* and *C. albicans.*[17,18] Also, CHX possesses substantivity, which is when dentin and other mineralized tissue can absorb and release it in sustainable fashion overtime.[19]

There has been an attempt to combine an antiseptic solution (CHX) with a decalcifying solution (10% CA) which results showing that the mixture did not alter the decalcifying capacity of the solution.[20] Previous study showed that the combination of CHX with CA had antimicrobial activity against the planktonic-grown *E. faecalis* and *C. albicans* while remaining smear layer removal efficacy.[21] However, there is no study on the antibiofilm effect of that mixed solution. Therefore, this study aimed to study the antibiofilm effect of CA-modified CHX against *E. faecalis* and *C. albicans* dual-species biofilm.

Materials and Methods

This experimental protocol was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University, Thailand (reference number 2/2019), and Institutional Biosafety Committee, Chiang Mai University, Thailand (CMUIBC0562004).

1. Sample preparation

1.1 Dentin slices preparation

Thirty-two human third molars were extracted and kept in a 0.1% (v/v) thymol solution after obtaining informed consent from the patients. Teeth with dental caries and restoration were excluded. The crown was separated from the root with a diamond disc (Intensive SA, Switzerland). Dentin slices were prepared from the crown with a precision sectioning saw (Isomet® 1000; Buehler Ltd, Lake Bluff, IL, USA) to a thickness of 1 mm. Then, the slices were shaped to a 6x6 mm square. Next, 800 and 1000 grit sandpapers were used to polish the dentin slice to ensure a smooth surface.

1.2 Sample sterilization

Dentin slices were treated with 3 solutions in an ultrasonic bath. First, 5 minute of 5.25% NaOCl was used for disinfection, then 1 minute of 17% EDTA was used for smear layer removal and followed by 5 minute of 5% sodium thiosulphate to neutralize the NaOCl. Later, samples were immersed in brain heart infusion broth (BHI broth; HiMedia, HiMedia Laboratories, India) and sterilized by autoclaving at 121°C at least 15 psi of pressure for 15 minute.

2. Preparation of *E. faecalis* and *C. albicans*

E. faecalis strain ATCC 29212 was streaked and cultured on brain heart infusion agar (BHI agar; HiMedia, HiMedia Laboratories, India) in an incubator (Memmert BE-200; Memmert GmbH.Co., Germany) under aerobic condition, at 37°C for 24 hours. Then, 3-5 colonies of the bacteria were transferred to the sterilized BHI broth. The broth was incubated at 37°C for 4 hours. The bacteria suspension was adjusted spectrophotometrically in phosphate-buffered saline (PBS) to $1x10^8$ colony-forming unit per milliliters (CFU/ml) using McFarland standard tubes no. 0.5.

C. albicans strain ATCC 10231 was streaked and cultured on sabouraud dextrose agar (SDA; HiMedia, HiMedia Laboratories, India) in an incubator under aerobic condition, at 37°C for 24 hours. Then, 5 colonies of the fungi were transferred to the sterilized BHI broth. The fungi suspension was adjusted spectrophotometrically in PBS to $1x10^6$ CFU/ml using McFarland standard tubes no. 0.5.

3. Bacterial-fungal biofilm formation

Dentin slices were placed in 6-well cell culture plates. *C. albicans* combined with *E. faecalis* at 1:100 ratio was mixed into BHI broth. Dentin slices were immersed in the broth in 6-well plates. The inoculum was incubated at 37°C for 14 days with 2 ml of the BHI culture medium replaced every 48 hrs. On day 14, the incubated dentin slices were rinsed with 2 ml of PBS. Then, two of the dentin slices were randomly selected to investigate under scanning electron microscopy (SEM) to confirm the formation of a bacterial-fungal biofilm.

4. Effect of CAmCHX against bacterial-fungal biofilm

The dentin slices were randomly assigned to 4 groups.

Negative control: PBS (n=6)
Group 1: 2% CHX (n=6)
Group 2: 10% CA (n=6)
Group 3: CAmCHX (n=6)

Biofilm-grown dentin slices were immersed in 2 ml of the experimental solution for 1 minutes, then rinsed off with PBS.

4.1 Microorganisms viability test using confocal laser scanning microscopy (CLSM)

Samples were stained with a viability fluorescent staining (LIVE/DEAD BacLight viability stain; Molecular probes, Eugene, OR, USA). Every sample was stained with 20 μM of SYTO 9 for 20 minutes, 60 μM of propidium iodide (PI) for 1 minutes, and rinsed with PBS for 1 minutes. The excitation and emission wavelength were 480/500 nm for SYTO 9 and 490/635 nm for PI, respectively. Samples were investigated under CLSM (Nikon Eclipse TE2000; Nikon Canada, Mississauga, ON, Canada) and observed under x20 lens. For every sample, two areas of dentin slices were randomly scanned in a format of 512 x 512 pixels. The images were processed and analyzed using Image J® 1.52n software (Wayne Rasband, National Institute of Health, USA). Cells with red and yellow staining represent dead cells, whereas those with green staining represent the total cells. The proportion of dead cells to total cells was calculated. One-way ANOVA and Tukey's multiple comparisons were used for statistical analysis using IBM SPSS Statistics software version 17 (IBM Corporation, Armonk, NY, USA) at the significance level $p<0.05$.

4.2 Biofilm microstructure evaluation using SEM

Eight biofilm-grown dentin slices (2 from each group) were fixed in 2.5% glutaraldehyde for 45 minutes. Serial dehydration was performed with absolute ethanol (50, 70, 85, 95, and 100%), each for 15 minutes. Later, samples were critical point dried and coated with gold-palladium particles. The biofilm microstructure evaluation was performed using SEM (JSM 3310LV; JEOL Ltd., Tokyo, Japan) at 1500x and 3000x magnifications.

Results

CLSM images (Figure 1) revealed an abundance of cells in both PBS and 2% CHX groups. The proportion of dead cells to total cells in the 2% CHX group was significantly higher than those in the PBS group ($p<0.05$). On the other hand, in 10% CA and CAmCHX groups, the remaining cells were less than the others ($p<0.05$). The proportion of dead cells to total cells was 77.82% for the 10% CA group and 58.91% for the mixed solution. All experimental groups resulted in a significantly higher proportion of dead cells to total cells compared to the PBS group ($p<0.05$). However, there was no significant difference between the proportion of dead cells to total cells in the mixed solution and 2% CHX groups ($p>0.05$) (Table 1).

Table 1: Proportion of dead cells to total cells and biofilm removal, investigated by CLSM and SEM.

Solutions	CLSM with viability staining	SEM
	Mean Cell Death (%)	Biofilm status
PBS	2.76^{a}	Intact
2% CHX	43.45^{b}	Intact
10% CA	77.82^{c}	Disrupted
CAmCHX	58.91^{bc}	Disrupted

Mean values represented with the same lowercase letters (row) are not significantly different according to each solutions ($p>0.05$).

Different superscript letters indicate statistical differences between groups ($p<0.05$).

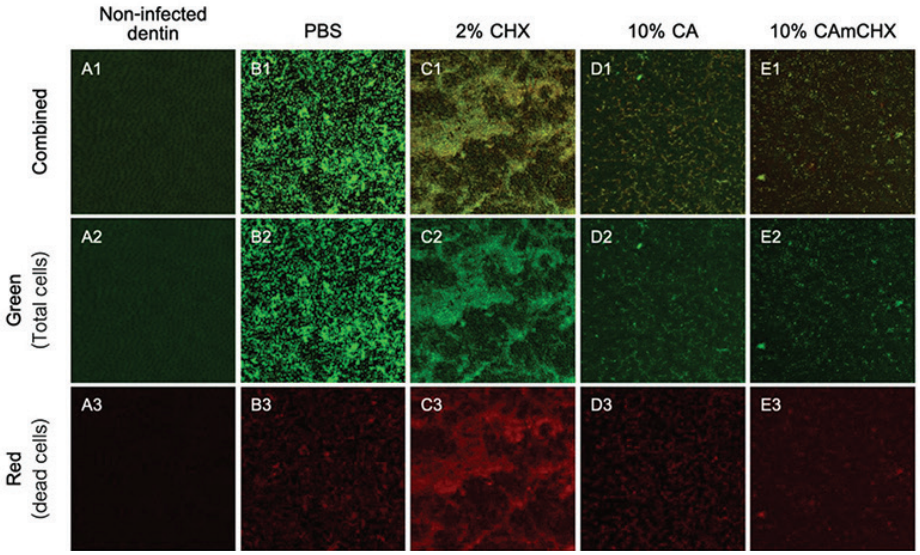

Figure 1: CLSM images (200x) showing the viability status of microbes on dentin slices after immersing in experimental solutions. (A) Non-infected dentin. (B) PBS group. (C) 2% CHX group. (D) 10% CA group. (E) CAmCHX group.

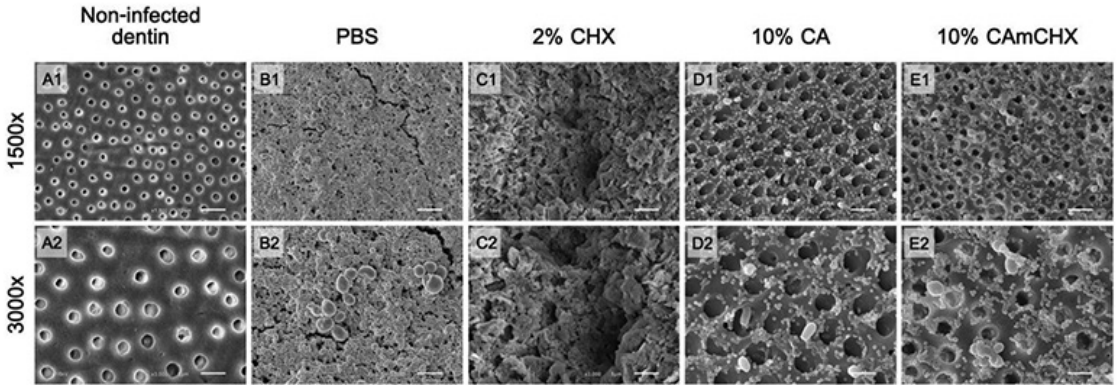

Figure 2: SEM images at 15,000x and 30,000x magnification showing the surface of dentin slices after immersing in experimental solutions. (A) Non-infected dentin. (B) PBS group. (C) 2% CHX group. (D) 10% CA group. (E) CAmCHX group. Smear layer and high density of biofilm layer completely covered the dentin surface in the control group (B) and 2% CHX group (C). There is less remaining biofilm observed in the 10% CA (D) and 10% CAmCHX groups (E). CLSM: confocal laser scanning microscopy, SEM: scanning electron microscopy, PBS: phosphate-buffered saline, CHX: chlorhexidine gluconate, CA: citric acid, CAmCHX: citric acid-modified chlorhexidine gluconate.

Similar results were also observed in SEM (Figure 2). There was a high density of biofilm layer on the dentin surface in PBS and 2% CHX groups. On the other hand, there was less remaining biofilm in the 10% CA and CAmCHX groups compared to other groups. In most samples of the 10% CA group, there was evidence of peritubular and intertubular dentin destructions (Figure 3).

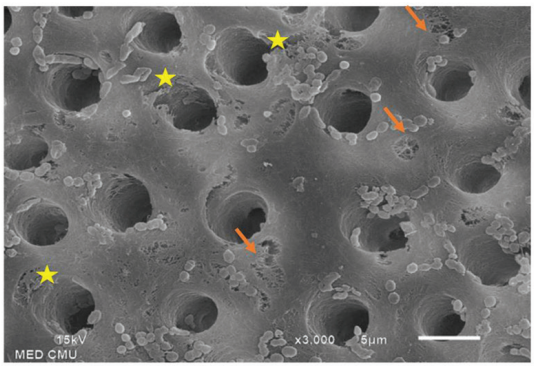

Figure 3: SEM image demonstrates that 10% CA caused the structural damage to the peritubular (stars) and intertubular dentin (arrows).

Discussion

This study evaluated the antibiofilm effect of 10% CA-modified 2% CHX against *E. faecalis* and *C. albicans* dual-species biofilm. The results showed that the solution

was able to kill and disrupt *E. faecalis* and *C. albicans* biofilm on dentin slices. Many studies on the antimicrobial efficacy of root canal disinfection used the planktonic culture method or monoculture biofilm model.[12,13] Endodontic infection, on the other hand, is polymicrobial infection.[3] *E. faecalis* can be found along with several other taxa, such as *C. albicans,* in root canal treated tooth, especially in persistent endodontic infection.[3,22] Microbial biofilm is more resistant to eradicate than planktonic microorganisms. *E. faecalis* biofilm is 1,000 times more resistant against phagocytosis, antibodies, and antibiotics.[23] Therefore, the authors selected a multi-species biofilm model in this study.

The multiple species of bacteria grown in a biofilm can enter the Viable but nonculturable (VBNC) state after nutrient deprivation and can be brought back to a culturable state afterward.[24] *E. faecalis* was hypothesized to enter the VBNC state as well.[5] Under a challenging environment, CLSM combined with viability staining could indicate the viability of organisms with greater accuracy than a culture-based method.[25] Therefore, this study selected a viability fluorescent staining instead of a culture method.

There are several *in vitro* studies demonstrating the eradication of *E. faecalis, C. albicans,* and other microorganisms using CHX.[26,27] By interacting with the cell membrane of microorganisms, CHX increases cell wall permeability, leading to the precipitation of cytoplasmic organelles. Moreover, the charged ions released from CHX can be absorbed by dentin, which provides protection against the colonization of microbes on the root canal dentinal wall. The absorption also resulted in a prolonged antimicrobial effect beyond the irrigating period.[9] On the other hand, CA and 17% EDTA demonstrated less antimicrobial effect against the two microorganisms *(E. faecalis* and *C. albicans)*. CA is one of the chelating agents. According to Chandra *et al.*[28], chelating agents can expose dentinal tubules allowing other irrigants to penetrate deeper into the tubules. As a result, microbes can be eradicated superiorly since microorganisms in the smear layer possessed the ability to withstand irrigants. From the viability test of the current study, the antimicrobial property against dual-species biofilm of CAmCHX was comparable to 2% CHX. However, CAmCHX disrupted biofilm structure better than 2% CHX. This might be the results from the demineralization and microbial cells inhibition via metal chelation of CA.[14,29]

CHX is considered a gold standard as a broad-spectrum oral disinfectant.[19] However, in biofilm removal, its efficacy is inferior to others. Clegg *et al.*[15] studied the effect of 6%, 3% NaOCl, 1%, 2% CHX, and BioPure MTAD on biofilm removal from the apical part of root dentin and revealed that 2% CHX could not disrupt the biofilm. This remaining biofilm can interfere with the root canal filling material, leading to voids and ultimately treatment failure. This study revealed that CAmCHX can remove biofilm. This finding is consistent with other studies on biofilm removal efficacy of CA from denture surface[30] and titanium implant.[31]

The chelating agent used in this study is CA. Malheiros *et al.*[32] compared the cytotoxicity of 17% EDTA, 10%, 15%, and 25% CA against fibroblast and found that there were no cell growth impairment nor changes in viability in CA groups. Moreover, CA produced less dentinal tubule enlargement on the root dentin surface than EDTA while exhibiting superior smear layer removal capability.[33] However, some samples in the 10% CA group of this study showed destruction of intertubular and peritubular dentin observed from SEM. This may be the result of the more acidic property of CA, which has a pH of 1.77, than CAmCHX, which has a pH of 2.29. This destruction of the dentin structure could jeopardize the seal between the root canal filling material and the root canal wall.[34,35]

This study chose 1 min immersion time to match the recommended irrigation protocol for a chelating agent to remove the smear layer before the final flush with an antimicrobial agent.[36] However, SEM images showed evidence of remaining biofilm in many specimens. This evidence highlights that a quick flush with a chelating agent might not be adequate for the complete eradication of the biofilm. This is consistent with the findings of Wang *et al.*[37], which stated that a longer irrigation time results in greater efficacy of microbial eradication from the infected dentinal tubule. Therefore, irrigating duration and antimicrobial efficacy of the irrigant could be important factors in the eradication of biofilm in the deeper dentinal tubules, which microbes reside. However, from the pilot study, the authors found that increasing exposure time with the chelating agent for more than 1 minute can lead to structural damage to the dentin. The destruction might be due to the more acidic property of the irrigant.

This finding corresponds with a study reported that the extension of irrigation time may increase the decalcifying effect on dentin.[34]

The limitation of this study was that the model was merely an *in vitro* simulation of the biofilm on dentin slices. Additional study in a root canal model with multi-species microorganisms is needed because root canal infection is caused by polymicrobial microorganisms.[23] There might be some mechanisms or relationships between those microorganisms which impairs the effectiveness of the solution. Additionally, studies on the cytotoxicity of the solution in humans are also needed.

Conclusions

CAmCHX had an antimicrobial effect against *E. faecalis* and *C. albicans*. Moreover, the the solution of CAmCHX could disrupt the *E. faecalis* and *C. albicans* biofilm on the dentin slices. Within the limitations of this study, CAmCHX had a significant antibiofilm effect against a dual species biofilm. Therefore, there is a potential for this solution to be used as a final rinse solution before root canal obturation.

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