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Antimicrobial Effect of a Lauric Acid on *Streptococcus Mutans* Biofilm.

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ABSTRACT

Background: The purpose of this study was to compare the antimicrobial activity of a synthetic fatty acid sodium laurate (Lauric acid) comprising dodecanoate fatty acids with chlorhexidine (CHX) or calcium hydroxide (CH) against S. mutans biofilm. **Methods:** *S. mutans* was grown on cover glass bottom dishes or human dentin disks for 3 days, and then treated with sodium laurate (20 μg/ml), non-functional fatty acid(sodium decanate, sigma Aldrich, C4151) ((NP, 20 μg/ml), CH (20 μg/ml), 1% CHX, or saline for 5 days at 37℃. On cover glass, live and dead microbials in the biomass were measured by the Film Tracer™ Biofilm viability assay, and observed by confocal laser scanning microscopy (CLSM). On dentin disk, normal, diminished, or ruptured microbials were observed by field-emission scanning electron microscopy (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:** Live/Dead Biofilm viability assay and CLSM demonstrated that sodium laurate treated biofilms had a significantly less bio-volume than CH, NP, and saline ($P < 0.05$), but had no significant difference from the CHX-treated group ($P > 0.05$). FE-SEM demonstrated that there was a marked decrease in aggregations of microbials and biofilm and wrinkled or ruptured microbials were frequently observed in the CHX and sodium laurate. **Conclusion:** Synthetic sodium laurate fatty acid exhibited significantly higher antimicrobial activity than CH by inhibiting microbial survival and biofilm growth against *S. mutans*, but had no significant difference compared to CHX.

Keywords: Lauric acid, Antimicrobial activity, *S. mutans* biofilm, dental caries.

INTRODUCTION

Streptococcus mutans, belong to commensal microbes, often colonize the human oral cavity and is a significant contributor to tooth decay of dental patients.[1] *Streptococcus mutans* (S. mutans) are commonly isolated from tooth-surface biofilm with dental caries.[2] After they invade dentinal tubules, colonize dentinal walls, and are considered to be acidophilic micro-environment called biofilms.[3] Furthermore, *S. mutans* frequently form biofilm that are more resistant to antimicrobial agent such as fluconazole.[4] The resistance of biofilms is due to nutrient limitation and slow growth, poor antibiotic penetration, adaptive stress responses, and formation of persister microbials. Actually, *S. mutans* contamination of the dentinal tubules could be the cause of endodontic treatment failure.[5]

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Chemo-mechanical instrumentation cannot completely eliminate microorganisms from the dentinal canal system.[5] The remaining microorganisms grow and multiply within the canals unless antimicrobial medicaments are used between appointments. Therefore, intracanal medicaments have been used to disinfect the dentinal canals and eradicate the remaining microorganisms. However, the most commonly used medicament, calcium hydroxide (CH), has been reported to be ineffective against *S. mutans*.[6] This may be due to the low solubility and diffusibility of CH , or the resilience of *S. mutans* in an alkaline environment.[6] Although chlorhexidine (CHX) has shown antimicrobial effects against *S. mutans*,[7] phenotypic resistance was exhibited by subpopulations of microbials within biofilms. Furthermore, antimicrobial antibiotics used to treat microbial infection, have shown the risks of developing resistance and host sensitization from repeated use.[8] Thus, a search for alternative preventative medicaments including innate antimicrobial fatty acids was initiated.

Antimicrobial fatty acids (AFAs) found in the oral cavity serve as host defense.[9] These include sodium laurate (Lauric acid), which are natural fatty acid, could be a useful component in a treatment for acne, but no clinical trials have yet been conducted to evaluate this potential benefit in

humans in inflamed tissues.[10] Among these, Lauric acid was found to have the strongest antimicrobial activity.[10] Since lauric acid is a saturated fatty acid with a 12-carbon atom chain, thus falling into the medium chain fatty acids, synthetic fatty acids were suggested as antibacterial agents. A synthetic lauric acid with fluorides was reported to have antimicrobial activity against *E. coli*.

Some previous studies demonstrated that a synthetic lauric acid (sodium laurate) has antimicrobial efficacy against both *E. faecalis* biofilms and multispecies biofilms,[11] but its antimicrobial activity is unknown. Therefore, the purpose of this study was to compare the antimicrobial activity of sodium laurate with CH and CHX against *S. mutans* biofilm.

MATERIALS AND METHODS

Effects of sodium laurate on *S. mutans* biofilm on dentin disks

Single-canaled 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, Seoul, Korea. Calculus and soft tissue on the dentinal surfaces were removed by an ultrasonic scaler, and the teeth were stored in sodium azide (0.5%, Sigma-Aldrich, St. Louis, MO) at 4°C. The canals were sliced into 500 m-thick cross sections using an Isomet precision saw (Buehler, Lake Bluff, IL). 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. *S. mutans* (ATCC 25175) were grown in yeast, malt media at 37°C until they reached mid-log phase (A600=0.1). Replicate dentin disks were incubated with microbial aliquots (300 µl/well, 6 X 10^6 microbials/ml) in 24-well plates for 3 days and treated with saline, non-functional fatty acid (NP, 20 µg/ml, Sigma), CH (20 µg/ml, DC Chemical Co Ltd, Seoul, Korea), 1% CHX (Sigma-Aldrich), or sodium laurate (20µg/ml, Tokyo chemical industry) for 5 days at 37 °C. The fatty acids were serially diluted in ethanol and trichloro-mono-fluoromethanol, and then applied to dentin disks. After incubation the dentin surfaces were examined by field-emission scanning electron microscopy (FE-SEM; S-4700, Hitachi, Tokyo, Japan).

LIVE/DEAD Biofilm viability assay

S. mutans mid-log phase cultures (3 ml/dish, 6 X 10^6 microbials/ml) were transferred to a cover glass bottom dish (SPL, Seoul, Korea) and incubated for 3 days, and then treated with sodium laurate, CH, CHX, NP, or saline for 3 days at 37℃. Each dish was aspirated to remove the planktonic *S. mutans* and washed gently with phosphate buffered saline (PBS). Finally, they were stained with the FilmTracer™ LIVE/DEAD Biofilm viability kit (Molecular Probes, Carlsbad, CA), which contains SYTO 9 and propidium iodide (PI) to stain live and dead microbials respectively. SYTO 9 stains both live and dead microorganisms in fluorescent green, whereas PI only stains the nucleic acids of microbials with damaged membranes and thereby identifies dead microbes. The stained *S. mutans* biofilms were examined by confocal laser scanning microscopy (CLSM, LSM 700, Carl Zeiss, Jena, Germany) with the X40 lens. CLSM images were acquired by using ZEN 2010 (Carl Zeiss) software at a resolution of 512 x 512 pixels with a zoom factor of 2.0. Each 2-dimensional (2D) image covered an area of 230.34 x 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.

Image and statistical analyses

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

RESULTS

FE-SEM observation of medicated S. mutans biofilm on dentin disks

A typical overview of *S. mutans* morphologies in the normal condition and different samples for 3 days is shown in [Figure 1]. When *S. mutans* biofilms were treated with medicaments for 3 days, there was a marked decrease in aggregations of microbials and biofilm in the CHX and sodium laurate groups [Figure 1]. There was intact biofilm in the NP and saline groups, reduced microbials in the CH group, some microbials in the CHX group, and very few microbials in the sodium laurate group. Additionally, wrinkled and ruptured microbials were frequently observed with sodium laurate treatment. The sodium laurate had disrupted *S. mutans* membranes and markedly inhibited their aggregation for the formation of biofilms.

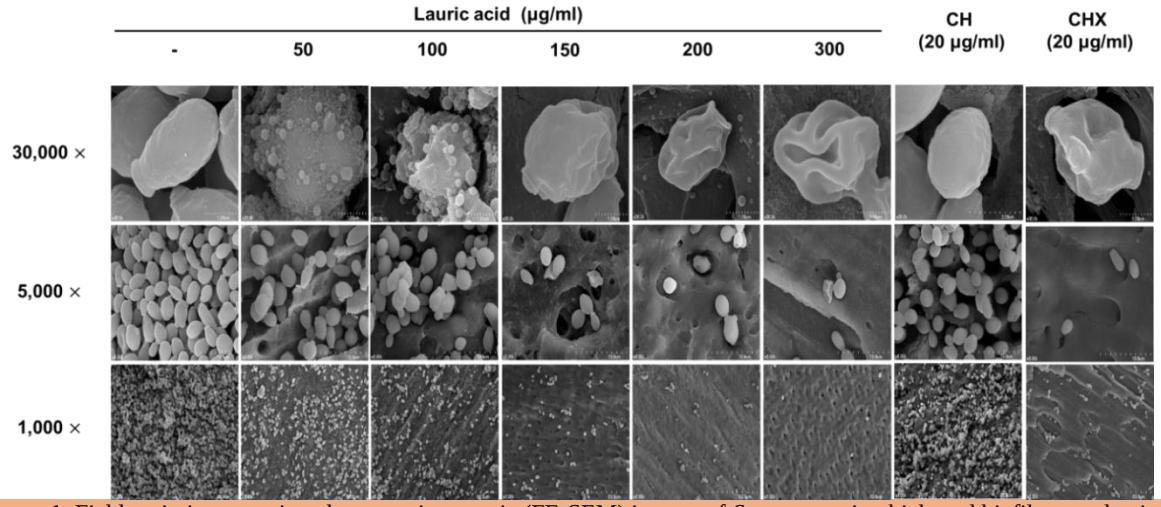

Figure 1: Field-emission scanning electron microscopic (FE-SEM) images of *S. mutans* microbials and biofilms on dentin. *S. mutans* (6×10^6 microbials/ml) was incubated on dentin for 3 days and treated with either sodium laurate (20 µg/ml), nonfunctional fatty acid (NP, 20 µg/ml), calcium hydroxide (CH, 20 µg/ml), chlorhexidine (CHX, 1%), or saline for 3 days. Biofilms and microbials were significantly reduced with CHX and sodium laurate (5000×, 1000×). Microbials that appeared normal (saline and NP) or wrinkled squashed walnut-shape (CH, CHX, & lauric acid) were seen (30,000×).

Live/Dead assay and CLSM observation of medicated *S. mutans* microbials

Biofilm formed on surfaces consists of bacteria, their secretion and extracellular polymeric substances (EPS). EPS produced by biofilms can act as a barrier to protect the bacteria from cellular immune response and antibiotics. Consequently, cells inside the biofilm have a much higher antibiotic tolerance compared with their planktonic counterparts, which makes them very difficult to eradicate. The biofilms that had been treated with CH, CHX or sodium laurate had a less proportion of live microbials and higher proportion of dead microbials than the NP or saline groups ($P < .05$), [Figures 2A]. In the saline or NP condition, extensive *S. mutans* biofilm was formed and reached about the 20µm thickness with visible dead and live bacteria. CLSM images show more dead *S. mutans* cells in the sodium laurate treated group with red color. Sodium laurate treated biofilms had a significantly less bio-volume than CH, NP, and saline ($P < .05$) and in sharp contrast, nearly no bacterial cells and no trace of biofilm were found on the high concentrated sodium laurate treated group, but had no significant difference from the CHX-treated group ($P > .05$), [Figure 2B].

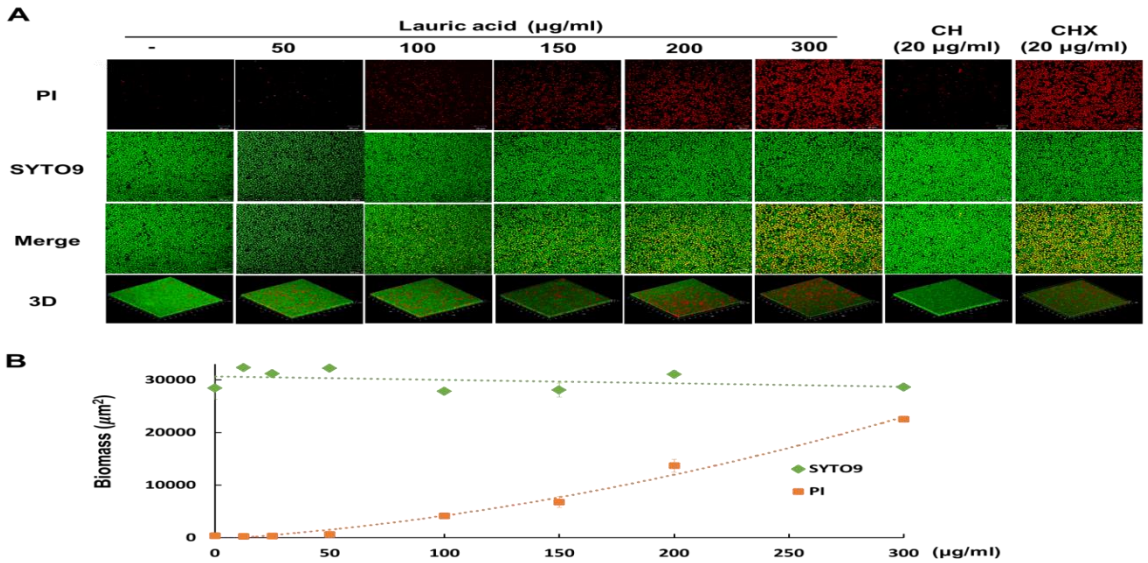

Figure 2: Bio-volumes (μm^3) of *S. mutans* biofilms on cover glass. *S. mutans* (6×10^6 microbials/ml) was incubated on cover glass for 3 days and treated with either sodium laurate (20 µg/ml), nonfunctional fatty acid (NP, 20 µg/ml), calcium hydroxide (CH, 20 µg/ml), chlorhexidine (CHX, 1%), or saline for 3 days. (A) Film Tracer LIVE/DEAD Biofilm Viability staining and examination by CLSM followed by 3-dimensional reconstructions, showed fewer live (green) microbials and some dead (red) microbials with CH, CHX, and sodium laurate. (B) BioImage_L software calculations showed significantly less bio-volume in the CHX and sodium laurate.

Dimensional Analysis of *S. mutans* biofilms

The biomass for the total population of microbial microbials appeared to be normally distributed across the z level plot (0-15 m). Their highest densities were at around 6 m in the CH, NP and saline [Figures 3A, 3B, & 3C], and at around 7-8 m in the CHX and sodium laurate treated biofilms [Figures 3D & 3E]. Dead (red) microbials increased in the sodium laurate treated biofilms.

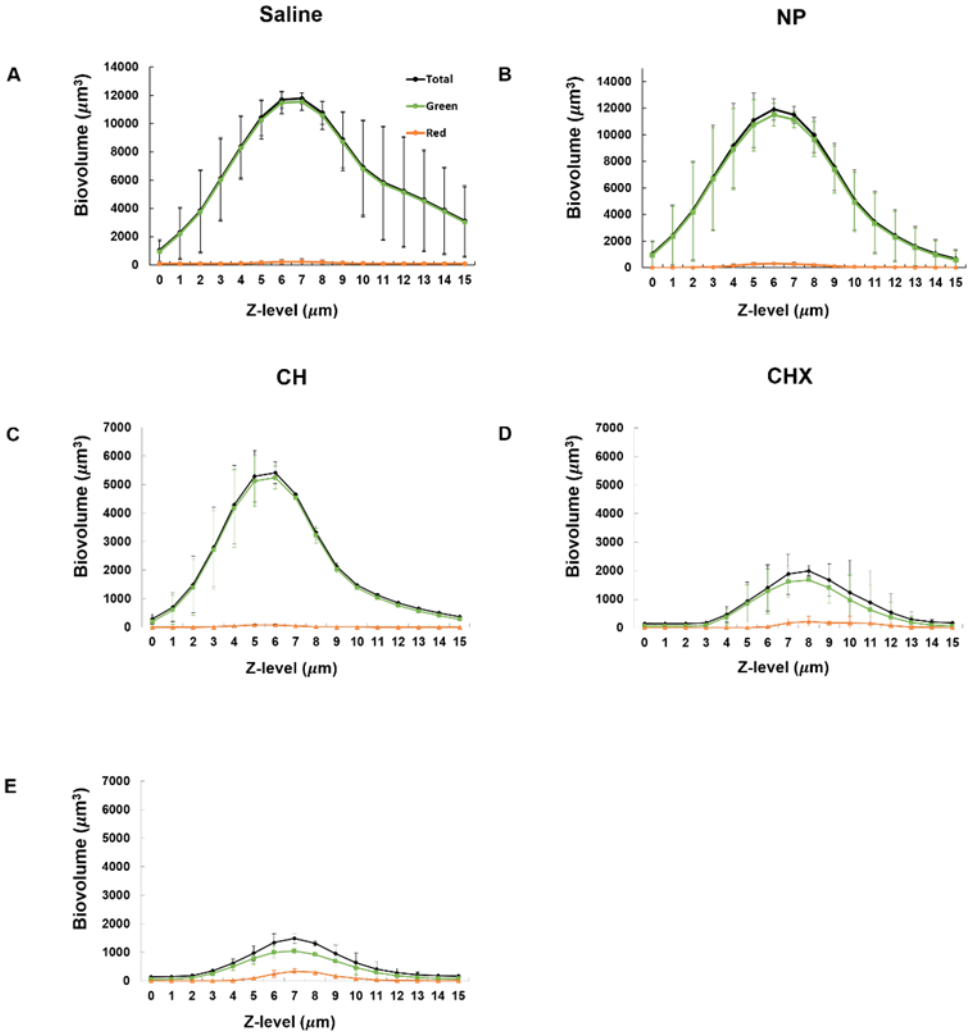

Figure 3: Biomass values of total population and green and red subpopulation corresponding to different z levels: (A) saline, (B) non-functional fatty acids, (C) CH, (D) CHX, (E) sodium laurate. The *S. mutans* microbials distributed throughout z levels. Any particular pattern depending on biofilm depth and medication was not observed.

DISCUSSION

Streptococcus mutans is one of the most important oral bacterial, which plays a major role in dental caries, bacteremia and consequently bacterial endocarditis among predisposed patient.[12,13] The present study is an experimental study done on 20 volunteers from the SGT Dental College. *Streptococcus mutans* is the organism chosen for the study as it is present in the oral cavity in the majority and it has an important role in the dental diseases.[14] Prevention of dental caries can be achieved by proper and regular tooth brushing and rinsing with mouth rinses containing antibacterial agent such as chlorhexidine, sodium hypochlorite are widely used as mouthwashes and irrigating agents, respectively, but this antibacterial agent is widely used have side effect such as cytotoxic to human periodontal ligament cells, inhibition protein synthesis and affect mitochondrial activity of these cells.[15] The antibiotics for prevention of dental caries is not recommended, since there is risk of development of bacterial resistant.[16]

This study compared the antimicrobial activity of synthetic sodium laurate fatty acid against *S. mutans* biofilms with CH and CHX, and showed it has better antimicrobial activity than CH and slightly better than CHX. Previously, lauric acid

was shown to be anti-inflammatory effect and reduced related cytokine expression.[10] However, the lauric acid has been never reported about its antimicrobial effects, even in the oral cavity pathogens. The sodium laurate as a special dodecanal fatty acid has the potential for greater antimicrobial activity and preventative effect for dental caries at a reduced cost.

FE-SEM showed the disruption and few aggregations of the *S. mutans* microbials in the sodium laurate and CHX-treated groups. Furthermore, CLSM evaluation showed that the proportion of dead microbes in the sodium laurate and CHX-treated biofilms were significantly higher than that of the CH. A previous study demonstrated that the antimicrobial activity of CHX was more effective than CH against *S. mutans* [17], which was consistent with the present study. This might due to the substantivity of CHX, which might prevent the *S. mutans* colonization as it did prevent the bacterial colonization on dentinal surface.[18]

Regarding the antimicrobial effect of CH, it had limited efficacy in killing *S. mutans* biofilm in our study, which is consistent with the previous reports. *S. mutans* could survive at a wide range of pH and the alkalinity of aqueous CH may not have an antimicrobial effect on *S. mutans*.[19] The antimicrobial efficacy of the CH was high in the first 24 h against *S. mutans*, whereas the efficacy gradually reduced after 72 h. This might be attributed to the dilution of the CH as time progressed or dentin buffering effect.[20] In addition, CH may provide the Ca^{2+} ions necessary for the growth and morphogenesis of *S. mutans*.[21] These mechanisms may explain why CH has been found to be ineffective against *S. mutans*.

Collectively the present results suggest that the sodium laurate fatty acid may be effective as an antimicrobial medicament. In addition, lauric acid had a very low cytotoxicity against host cells.[22] Its antimicrobial efficacy and range of target microorganisms could be modulated by designing analogs of lauric acid,[23] and could create synergy when incorporated with other antimicrobials and disinfectants such as CHX. Furthermore, the sodium laurate has a low viscosity that will prolong its contact with dentinal tubules and dentinal canal walls, and promote its delivery to inaccessible isthmuses, canal fins, and curved canals. Taking together the present anti-inflammatory effect with the antimicrobial effects against endodontic pathogens,[10] the sodium laurate fatty acid could be applied as an injectable intracanal medicament for therapy-resistant/persistent dentinal canal infection or for endodontic regenerative procedure of infected immature permanent tooth.

CONCLUSION

The sodium laurate exhibited significantly higher antimicrobial activity than CH by inhibiting microbial survival and biofilm growth against specific pathogen *S. mutans*, but had no significant difference compared to CHX. The obtained data highlight the potential of using lauric acid as an alternative treatment option to the antibiotic therapy of dental caries.

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