



Title	An extensive description of the microbiological effects of silver diamine fluoride using an oral in situ model
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Abstract of Thesis

Name (Klanliang Kittipit)	
Title	An extensive description of the microbiological effects of silver diamine fluoride using an oral in situ model (フッ化ジアンミン銀がin situデンタルバイオフィルムに及ぼす微生物学的影響の多面的解析)
Background and Objective: Over the past decades, studies have demonstrated the vital role of biofilms and their impact on oral health caused by environmental changes that lead to a shift from symbiotic to dysbiotic state. This process eventually results in infectious oral diseases, such as periodontitis and dental caries. Root caries are considered a major issue in middle-aged and elderly individuals. A root carious lesion originates from exposed root surfaces that are vulnerable to acid attack by bacteria residing in root dentin biofilms. Consequently, several professional approaches have been recommended, such as the use of various fluoride products, including fluoride toothpaste and silver diamine fluoride (SDF). The clinical effectiveness of SDF has been investigated in both coronal and root caries. <i>In vitro</i> studies have demonstrated the antibacterial efficacy of SDF on cariogenic biofilms, including its ability to inhibit demineralization and protect dentin collagen from degradation. However, the impact on the microbial community after SDF application on root surface dentin has not been extensively studied. Therefore, this study aimed to investigate the microbiological effects of SDF on dental biofilms on demineralized root dentin using <i>in situ</i> model. Materials and Methods: 1. <u>Volunteer recruitment:</u> Ten healthy volunteers, aged 26 to 31 years, were recruited. The study design was approved by the Ethics Committee of Osaka University Graduate School of Dentistry (H29-E17-2). 2. <u>In situ model and experimental design:</u> An <i>in situ</i> intraoral device, consisting of left and right pieces, was designed with eight rectangular slots for positioning of dentin specimens to cultivate biofilms. Root dentin slabs of bovine incisors were prepared and equally divided into two groups: control and SDF groups. The surfaces of all the slabs were demineralized using 20% citric acid. The demineralized dentin slabs were then used as substrates for biofilm formation. Slabs in the SDF group were treated with 38% SDF for 4 min, whereas the control group comprised untreated slabs. Volunteers wore the devices with dentin slabs, and the biofilms formed were collected daily from days 1 to 4. 3. <u>Viable cell count:</u> The biofilms on dentin slabs were removed using an ultrasonic machine and then cultured on Columbia blood agar plates supplemented with 5% sheep blood. The plates were incubated under aerobic and anaerobic conditions for 48 h prior to counting of the colonies. 4. <u>Quantification of biofilm-forming cells:</u> DNA was extracted from biofilm samples. Real-time PCR was performed using a 7500 Fast Real-Time PCR system with universal bacterial primers. Standard curves were generated, and the number of biofilm-forming cells was assessed. 5. <u>Confocal microscopy:</u> The biofilms were labeled with the LIVE/DEAD cell staining kit and observed using a confocal laser scanning microscope. Additionally, the 3D images were reconstructed using the Imaris software. The thickness of the biofilms and the volume of dead and live bacteria were assessed. 6. <u>SEM investigation:</u> The dentin slabs with biofilms were immersed in 50% Karnovsky's solution before serial dehydration was performed using ethanol solutions. Thereafter, the specimens were subjected to freeze drying and sputter coating with platinum. The microstructures of the biofilms were then observed using SEM.	

7. The 16S rRNA gene sequencing analysis: The bacterial profiles were identified and compared between the groups using the Illumina MiSeq sequencing platform and QIIME. The bacterial diversity was analyzed and compared within and between groups to investigate the differences in microbial communities.

8. Quantification of commensal and caries-associated species: To screen for specific streptococcal species associated with healthy and dental caries, real-time PCR with species-specific primers for streptococci was used to quantify the number of bacterial cells in the DNA samples.

Results and Discussion:

1. Quantification using viable cell count and real-time PCR: The average CFU count was significantly higher in the control group than that in the SDF group under both aerobic and anaerobic conditions. Similarly, the total number of cells in the control group was greater than that in the SDF group. These results indicate that SDF has an inhibitory effect on dental biofilm formation.

2. Confocal microscopy and SEM: Comparisons between the two groups revealed a decreased number of cells and reduced biofilm thickness in the SDF group. Additionally, the CLSM images showed numerous dead cells enclosed. The result of ultrastructural characteristics of biofilms demonstrated under SEM showed the co-aggregation of various bacteria and the presence of matrix-like structures after 48 h of the experiment in control group's biofilm indicating the actual maturation of dental biofilm. While in SDF group, biofilms were found scarce on the dentin surface treated with SDF, and the bacterial composition was less complex. According these observations suggests that the natural maturation of dental biofilm was disturbed when tooth surface was treated with SDF solution.

3. A 16S rRNA gene sequencing analysis: Across all samples, 14 phyla, 21 classes, 33 orders, 58 families, and 90 genera were detected. Among the 20 most abundant taxa, *Streptococcus* was found to be more abundant in the SDF biofilm than in the control from day 2 to day 4 of the experiment. Meanwhile, *Actinomyces*, which exhibited a lower abundance in the SDF group, was also found more frequently in the SDF group throughout the experiment. An analysis of bacterial diversity demonstrated that there was no statistical difference in the alpha diversity indices within the experimental groups or time points. Furthermore, there was no distinct clustering of data on the PCoA plot of beta diversity based on Bray–Curtis distance; this result was confirmed using the PERMANOVA test. Evaluation of the relative abundance suggested that the majority of genera were common in the control and SDF groups, while *Streptococcus* was the most prominent genus in both groups. However, the abundance of *Streptococcus* was higher in the SDF group than that in the control group. The analysis of bacterial diversity indicated that the global structure of microbial community of dental biofilms on demineralized dentin were not changed when dentin slabs were pretreated with SDF.

4. Quantification of commensal and caries-related species: All streptococcal species showed comparable numbers of bacterial cells in the SDF biofilm, which remained stable until day 4, whereas the number of cells of streptococci in the control group continued to increase over time. This suggests the effect of SDF may not specifically have an effect on certain species of *streptococcus*.

Conclusion:

The application of SDF to demineralized dentin limited the growth of dental biofilms and increased the proportion of dead bacterial cells. SDF did not distinctly alter the microbiome diversity on demineralized dentin after 4 days. In addition, the relative abundance of specific streptococci that were associated or not with root caries was similar between the groups. The noticeable decrease in the biomass of biofilms grown on SDF-treated dentin may potentially influence the efficacy of SDF in preventing and arresting caries on root dentin over a result of the change in bacterial composition.

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨 本研究では、根面う蝕に対するフッ化ジアンミン銀の効果を探求することを目的として、脱灰根面象牙質上に形成した <i>in situ</i> デンタルバイオフィルムへのフッ化ジアンミン銀の微生物学的影響を多面的に検討した。 その結果、フッ化ジアンミン銀はバイオフィルム細菌叢の多様性を変化させないが、初期付着抑制によりバイオフィルム量を有意に減少させ、抗バイオフィルム効果を発揮することが明らかとなった。 以上の研究成果は、フッ化ジアンミン銀が根面う蝕の発症予防と進行抑制に寄与する科学的根拠を提供するものであり、本研究は博士（歯学）の学位授与に値するものと認める。		