Effects of silver diamine fluoride on dentine carious lesions induced by *Streptococcus mutans* and *Actinomyces naeslundii* biofilms

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Abstract

Background: *Silver diamine fluoride (SDF) has been shown to be a successful treatment for arresting caries. However, the mechanism of SDF is to be elucidated.*
Aim: To characterize the effects of SDF on dentine carious induced by Streptococcus mutans and Actinomyces naeslundii.

Design: *Thirty-two artificially demineralized human dentine blocks were inoculated: 16 with S. mutans and 16 with A. naeslundii. Either SDF or water was applied to 8 blocks in each group. Biofilm morphology, microbial kinetics and viability were evaluated by scanning electron microscopy, colony forming units, and confocal microscopy. The crosssection of the dentine carious lesions were assessed by microhardness testing, scanning electron microscopy with energy-dispersive x-ray spectroscopy and Fourier transform infrared spectroscopy.*

Results: *Biofilm counts were reduced in SDF group than control (p<0.01). Surfaces of carious lesions due to S. mutans were harder (p<0.05), in S. mutans group, Ca and P weight percentage after SDF application than after water application (p<0.05). Lesions showed a significantly reduced level of matrix to phosphate after SDF treatment (p<0.05).*

Conclusion: *Present study showed that SDF posses an anti-microbial activity against cariogenic biofilm of S. mutans or A. naeslundii formed on dentine surfaces. SDF slowed down demineralization of dentine. This dual activity could be the reason behind clinical success of SDF.*

Introduction

The simplicity and affordability of SDF treatment has gained much attention in the past decade. Clinical trials showed that SDF prevented and arrested coronal caries in primary teeth in preschool children[1] and in permanent teeth in older children [2]. A 2-year randomized controlled trial reported that SDF prevented and arrested root caries [3]. The effect of SDF has been reported in 1970s (Yamaga et al., 1972), However, there are more accurate tools to investigate this effect on dental hard tissue and on biofilms. Recent reviews concluded that SDF is a safe, effective, efficient, and equitable caries-preventive agent and that it seems to meet the criteria of both the World Health Organization Millennium Goals and the United States Institute of Medicine's criteria for 21st century medical care [4, 5]. Despite studies suggested SDF could avoid enamel caries progress into dentine [6] and arrested established dentine caries [Chu et al., 2002], laboratory study to support the effectiveness of SDF in preventing and arresting caries is scant. In addition, very few studies have reported the effects of SDF on mineral content and microbial biofilm formation on dentine. Such evidence is needed to substantiate the findings of clinical trials testing SDF's effects on dentine caries, and to investigate the mechanisms by which SDF acts.

Some laboratory studies have revealed aspects of the mechanism by which SDF has anticariogenic properties when applied to dentine and enamel. For example, SDF significantly reduced demineralisation in sound bovine enamel and dentine [7], and SDF increased enamel surface micro-hardness and reduced enamel surface mineral loss [Delbem et al., 2006]. Another study found dentine of human primary teeth in which caries had been arrested by SDF had a greater microhardness than normal dentine [8]. After demineralised dentine had been pretreated with a monoculture of *Streptococcus mutans,* topical treatment with SDF and potassium iodide (KI) reduced *in vitro* caries development and inhibited surface biofilm formation [9]. The same SDF/KI treatment also reduced the permeability of demineralised dentine to *S. mutans.* On its own, SDF prevented the growth of *S. mutans* biofilms on both demineralised and non-demineralised dentine [9, 10].

Previous studies reported in the English literature have thus mainly focused on SDF's effect on tooth tissue. The few that have examined effects of SDF on biofilms used only *S. mutans* as the only bacterium [10]. Since *S. mutans* is the most predominate bacteria related with caries [11]. *A. naeslundii* was highly associated with root caries [12]. Hence, this study aimed to investigate the anti-microbial effect of SDF on *S. mutans* and *A. naeslundii* biofilms as well as the mineral content of dentine caries lesions. It is of noteworthy that mono-species biofilms are very different from complex *in vivo* multispecies plaque biofilms in both survival and pathogenic potential. The null hypothesis of the study was that SDF has no anti-microbial effect on *S. mutans* and *A. naeslundii* biofilms formed and has no effect on the mineral content of dentine caries lesions. The outcomes measures are colony forming unit and dead/live ratio of the biofilms; and microhardness, Calcium and Phosphate content and matrix to phosphate ratio of the carious lesions.

Materials and methods

Sample preparation

This study was approved by a local Institutional Review Board (IRB UW08-052) and patients consented before the study. Extracted human third molars that were deemed sound were stored in 1% sodium azide at 4°C. Dentine blocks of $2\times2\times4$ mm^3 were prepared as described in a previous study [13] and divided in to 2 groups. We assumed the mean lesion depth before and after the 7-day of demineralization challenge were 100 μm and 150μm and the common standard deviation was 35 μm. The sample size was at least 8 in each group with power at 0.80 and α=0.05. A total of 32 dentine blocks were selected and examined under a stereomicroscope (×10 magnifications) to ensure they had no cracks, hypoplasia, or white spot lesions. Half of the surface of each block was coated with two layers of an acid-resistant nail varnish (Clarins, Paris, France) to serve as an internal control. To facilitate the development of carious lesions by cariogenic bacteria, each dentine block was incubated in an acidified buffer containing 50 mM acetic acid, 2.2 mM KH_2PO_4, and 2.2 mM $CaCl_2$, at pH 4.4, for 96 hours at 23°C [14, 15]. The blocks were then sterilized with ethylene oxide (Amsco Eagle 2017 EO sterilizer; STERIS, Mentor, USA) for 16 hours [16].

Actinomyces naeslundii American Type Culture Collection (ATCC) 12014 and *S. mutans* ATCC 35668 were cultured on blood agar plates at 37°C for 2 days anaerobically. A single colony was picked from each plate to prepare 24-hour broth cultures in basal medium supplemented with 5% glucose (BMG medium) at 37°C under anaerobic conditions [17]. After centrifugation, cell pellets were harvested and washed twice with phosphate buffered saline (PBS). Bacterial suspensions were then prepared in BMG to a cell density of McFarland 4 (10^9 cells/mL).

A 300µL aliquot of each bacteria was inoculated on each demineralised dentine block (8 blocks for each bacterium) sitting in 1mL BMG in a well of a 24-well plate. The plate was placed in an anaerobic chamber with 95% nitrogen and 5% carbon dioxide for 7 days to allow bacterial infiltration and formation of artificial caries lesions. The 7-day provides also allowed maturation of the biofilm [18]. The medium was refreshed daily without disturbing the specimen surface. After 7 days' incubation, 8 blocks in each bacteria group underwent topical application of 38% SDF solution (Saforide; Toyo Seiyaku Kasei Co. Ltd., Osaka, Japan) on exposed surfaces with a microbrush. The mean (±SD) amount of SDF applied was 0.22 mg ± 0.07 mg (or 8.8 µg ± 2.8 µg fluoride), as estimated by calculating the difference of the microbrush before and after application. The other 8 blocks in each group were treated with distilled water as a control. After treatment, all the dentine blocks were returned to the 24-well plate and placed on an incubator-shaker (Incubator-shaker 3525; Labline, Mumbai, India) set at at 75 rpm inside the anaerobic chamber for 7 days at 37°C.

Study of biofilms

Growth kinetics of the mono-species biofilm for 7 days was assesssed by determining bacterial counts in colony-forming units (CFU). For this step, serial 10-fold dilutions of homogenized biofilm samples in 1% PBS were plated in duplicate with a spiral plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA) onto horse blood agar (Defib Horse Blood; Hemostat Laboratories, Dixon, USA). Since fluoride ions might react with SiO_2 and cause inaccurate pH reading by a pH sensor, thus this study used pH test paper (Macherey-nagel, Düren, Germany) to measure the resting pH of the biofilm. The pH paper showed pH

value at 0.5 interval from <4.5 to >7.5 (8 categories).

Scanning electron microsocpy (SEM) was used to examine the topographical features of the biofilm. In preparation for SEM [19], biofilm samples were rinsed in 4% (vol/vol) formaldehyde followed by 1% (vol/vol) PBS; they were then placed in 1% osmium tetroxide solution for 60 min. Samples were washed in distilled water and dehydrated in a series of ethanol solutions at increasing concentrations (70% for 10 min, 95% for 10 min, and 100% for 20 min). Samples were then dried in a desiccator and sputter-coated with gold. The surface topographies of biofilms were studied under SEM (Leo 1530, LEO, Oberkochen, Germany) at 12 kV in high-vacuum mode.

Confocal laser scanning microscopy (CLSM) was used to study the viability of bacteria in biofilms on dentine carious lesions. Biofilms were labeled *in situ* using two fluorescent probes: PI and SYTO-9 (LIVE/DEAD BacLight Bacterial viability kit; Molecular Probes, Eugene, OR, USA). The red PI probe labels dead cells whereas the green SYTO-9 probe labels live cells. Dentine blocks were incubated in the dark for 30 min after labeling [20]. Thereafter, 4 cellular images of each biofilm specimen were obtained using CLSM (Fluoview FV 1000, Olympus, Tokyo, Japan) and examined using special image analysis software (Image J; National Institutes of Health, USA). The red-to-green ratio was calculated to indicate the ratio of dead-to-live bacteria on the anti-microbial effect of the therapeutic agent.

Study of hard tissue

Each dentine block was sectioned vertically, midway across the demineralisation surface. One half of the specimen was used for Knoop microhardness testing as well as elemental calcium (Ca) and phosphate (P), by scanning electron microscopy and energy-dispersive x-ray spectroscopy (SEM-EDS). The other half was used for Fourier transform infrared spectroscopy (FTIR) to evaluate any change in matrix to mineral content.

The cross-sectional surface of each dentine block was first polished with a sliding microtome under distilled-water irrigation (Leica 2500 SM; Ernst Leitz Wetzlar, Wetzlar,

Germany). The block was then subjected to Knoop microhardness testing (Leiz Microhardness Tester; Ernst Leitz Wetzlar, Wetzlar, Germany) with a load of 10 gf (98×10^{-3} N) for 10 s at each test point. Microhardness was determined at 25 μm below the surface of demineralisation, in increments of 50 μm for both exposed (test) and varnished (control) sides. Five sets of indentions were made on each specimen on parallel tracks approximately 150 to 200 μm apart, and Knoop microhardness measurements were made using computer software (Leica QGo-Applet Runner; Ernst Leitz, Wetzlar, Germany). The mean of the five sets of microhardness measurements at each section depth was recorded. To avoid variations of microhardness of different samples, internal control of the sound part in each dentine block was used for comparison. The low relative microhardness value represents a low value in microhardness [8].

The mineral content as levels of Ca and P of dentine lesions was analyzed by EDS (model 7426; Oxford Instruments, Oxford, UK) under SEM (Leo 1530 Gemini, Oberkochen, Germany). Elemental analysis was performed along a vertical line starting at 25 μm below the demineralisation surface and progressing at depths of 50 μm. Five line-scans were performed, and the mean Ca and P weight percentages and Ca:P ratio were calculated.

Changes in the content of the matrix (mainly type I collagen) to mineral (phosphate) content of dentine lesions were analyzed with a Bio-Rad FTIR UMA-500 machine (Bio-Rad Laboratories, Hercules, CA, USA), with infrared radiation ranging from 650 to 4000 cm^{-1} in wavelength number. Spectra for demineralised dentine lesions (n=4 for each bacteria group) were obtained by the average acquisition of data at the spatial resolution achieved with a 100×100 $μm^2$ aperture over the lesion surface. The spectrally derived matrix-to-mineral ratio was defined as the ratio of the area of absorbance of the protein amide I peak between 1585 and 1720 cm^{-1} to the area of absorbance of the HPO_4^{2-} peak between 900 and 1200 cm^{-1}. The log value of the [amide I: HPO_4^{2-}] absorbance ratio was then used as an indicator of the extent of demineralisation of dentine due to the carious activity of the biofilm [21].

Statistical analyses

All data will be assessed for a normal distribution using Shapiro-Wilk test for normality

(p>0.05). The *t* test was used to compare the pH values of biofilms, ratios of demineralised-to-sound dentine microhardness, Ca and P weight percentages, Ca:P ratios, and log [amide I:HPO$_4$$^{2-}$] ratio between SDF-treated and control groups at the same lesion depth. All analyses were conducted using SPSS version 17 software (SPSS Inc., Chicago, Illinois, USA). The cutoff level of significance was taken as 5% for all analyses.

Results

Biofilm characteristics

After SDF application treatment and growth for another 7 days, the bacterial counts decreased to zero in the biofilms of both *S. mutans* and *A. naeslundii* (Table 1). Very few live bacteria were detected in the two biofilm groups. In contrast, confluent growth of live *S. mutans* and *A. naeslundii* and high CFU counts were observed in the control groups. At the end of the experiment, pH values in the SDF treatment groups were was higher than those in the control groups.

In the SDF treatment groups, round particles of about 0.5 to 1μm that were visible in both *S. mutans* groups and *A. naeslundii* in SEM images (Figures 1a and 1c, respectively) were confirmed to be silver by EDS. In CLSM images, all bacteria present in the biofilm fluoresced red in SDF treatment group, indicating that the bacteria were dead after SDF application. In control groups, *S. mutans* and *A. naeslundii* formed thick, confluent biofilms. *S. mutans* biofilms contained long bead-like chains, a feature that is not seen under planktonic mode (Figure 1). *A. naeslundii* biofilms were seen as typical "Y"-shaped branched networks (Figure 1). The dead-to-live ratios from CLSM images (Figures 2), which indicate strength of anti-microbial effect, were significantly higher after SDF treatment than after water treatment in both *S. mutans* and *A. naeslundii* biofilms (p<0.01 and p<0.05, respectively; Table 1). The pH value of both *S. mutans* and *A. naeslundii* biofilms in the control groups were between 4.5-5.0, and the value increased to 6.0-6.5 with SDF treatment.

Hard tissue characteristics

The outer surface up to 125 μm of the dentine carious lesions due to *S. mutans* and *A. naeslundii* in the SDF subgroup was significantly harder than in the control subgroup (p<0.05; Figure 3 & 4).

In the dentine blocks bearing *S. mutans* biofilms, SEM-EDS revealed that both Ca and P weight percentages were higher after SDF application than after control treatment at 25 μm but not the other measured depths from the surface (Ca, p<0.05 and P, p<0.05; Figure 5). However, no significant difference of the Ca:P ratio in all measured depths from the surface (25 to 225 μm) was found (Figure 6). In addition, no significant differences in Ca and P weight percentages or Ca:P ratio were found between the SDF and control subgroups of dentine carious lesions caused by *A. naeslundii* biofilms (Figure 7&8).

The FTIR spectra of sound dentine and artificial caries lesion were shown in Figure 9. For the dentine, the absorbance for amide I occurred at 1585-1720^{-1}, and that for HPO_4^{2-} was from 900-1200cm^{-1}. The intensity of the phosphate band was strong in sound dentine but was weak in demineralised dentine; amide I band showed a litter bit higher in demineralised dentine than that in sound dentine. Interspecimen variation between study groups was large, therefore, all comparison were made with internal controls. The values of log [amide I: HPO_4^{2-}] are showed in Table 4. In all the three groups, the log [amide I: HPO_4^{2-}] ratio decreased after SDF application, with statistically significant reductions being found in *S. mutans* and *A. naeslundii* groups.

Discussion

This study provides essential information on the cariogenic effect of two types of mono-species bacterial biofilms on dentine and the anticariogenic effect of SDF. This provided useful information for the subsequent *in vitro* study with consortium or real saliva as inoculums. By using colony counts and CLSM, we have shown that SDF has a significant antimicrobial effect against both *S. mutans* and *A. naeslundii* biofilms. The SEM and EDS results revealed

precipitated silver in the biofilm.

Yamaga et al. (1972) considered the combination of silver and fluoride ions may prevent both calcium and phosphate ions from being lost, The mode of SDF action was suggested to be related to its reaction with calcium hydroxyapatite to form CaF_2 and Ag_3PO_4, for this reason, SDF was considered to be a better anticaries agent than silver nitrate or sodium fluoride [22]. Wu et al.[23] demonstrated silver has anti-bacteria effect and prevented biofilm formation. Silver can interact with sulfhydryl groups of proteins and with DNA, thereby altering hydrogen bonding and inhibiting respiration, DNA unwinding, cell-wall synthesis, and cell division [5].

The two bacteria strains used in this study belong to Streptococci and Actinomycetes, and were used because they are associated with dentine caries and can form mono-species biofilms (unlike, for example, *Lactobacillus acidophilus*). *S. mutans* bacteria are the most important cariogenic pathogens, as they are highly acidogenic and produce short-chain carboxylic acids, such as acetic acid, which dissolve dental hard tissue. Additionally, *S. mutans* ferments sucrose and produces extracellular polysaccharides that enhance bacterial adherence to tooth surfaces to facilitate biofilm formation. *A. naeslundii* is regarded as an important organism in early root caries development and has a pathogenic potential in root caries [24]. However, mono-species biofilms in a microplate system are very different from complex *in vivo* multispecies plaque biofilms in both survival and pathogenic potential. Therefore, the results cannot be extrapolated to the *in vivo* situation, and caution should be exercised in their interpretation.

The antimicrobial and anticariogenic effects of SDF were realised as a preservation of dentine content in both *S. mutans* and *A. naeslundii* samples. Microhardness testing showed that the surface of the dentine carious lesions was significantly harder in the subgroup treated with SDF than in the controls. A previous study also found that dentine carious lesions arrested by SDF had a significantly higher microhardness than normal dentine and appeared "hard" to clinical probing [8]. Measuring hardness has been shown to be a reasonable method of examining the mineral content of dentine with caries or arrested caries, by providing indirect

evidence of mineral loss or gain according to indentation depth [8, 25, 26]. SDF might directly inhibit biofilm growth and the fluoride might also precipitate as insoluble calcium fluoride, which could react with hydroxyapatite in dentine. Furthermore, the presence of calcium fluoride would make dentine more resistance to acid attack.

X-ray microanalysis is a common method of studying dental hard tissue and a variant method is EDS [27-29], which is used for elemental analysis at the ultrastructural level. The principle of EDS is based on energy emitted as x-ray photons when electrons from external sources hit the atoms in a material, with the x-rays being characteristic of each element. Studies of EDS have found that Ca and P levels are significantly higher in sound dentine than demineralised dentine [27-29]. We also found that Ca and P weight percentages in the outer 25 µm of dentine carious lesions due to *S. mutans* were significantly higher with SDF application than without. The reduced Ca and P weight percentages in the outer surface of the carious dentine controls indicates a loss of mineral density [30].

The Ca:P ratio reflects the mineral composition of the crystal lattice and may be used to help identifying certain materials. The ratio varies in biomineralised tissues, and for dentine it is between the values of 1.7 and 2.4 [31, 32]. Some researchers have suggested that the percentage loss of phosphate is always greater than the percentage loss of calcium in demineralised dentine, and thus the Ca:P ratio of dentine will be reduced by cariogenic biofilm challenge [29]. A recent study reported that the Ca:P ratio of sound dentine is 1.92 ± 0.18, whereas that of demineralised dentine is 2.01 ± 0.29 [30]. Moreover, the difference in Ca:P ratio between sound and demineralised dentine may due to a change in the crystal lattice during the demineralisation process of dentine. In this present investigation, the Ca:P ratio in the outer 25 µm of demineralised dentine was higher than in mineralized dentine. SDF can react with hydroxyapatite and form fluoroapatite and insoluble silver phosphate, which may contribute to the variations of Ca/P ratio. Moreover, a different calcium phosphate from hydroxyapatite such as amorphous calcium phosphate might have been produced. In amorphous calcium phosphate, the Ca/P ratio is variable, which would also explain the variations of the measured Ca/P ratio [30]. The large variations of the ratio could not give a conclusive finding in this study.

Dentine blocks in this study were first demineralised to facilitate bacterial challenge to simulate dentine caries. Our pilot study demonstrated acid demineralisation before bacterial challenge is essential to facilitate a noticeable lesion within 1 week of cariogenic biofilm challenge. Bacterial enzymes generated by biofilm challenge would cause destruction of dentine; destruction could also be mediated by acid-activated matrix metalloproteases present in dentine. Thus, dentine carious lesions developed in this study are in fact an artificial carious lesion that may behave as actual caries-like lesions. Although chemical acid dissolves the mineral phase of dentine it does not have a major effect on the matrix because the surface is protected by apatite crystallites. During biofilm challenge, proteolytic enzymes that are liberated by oral bacteria then destroy the organic matrix so that apatite crystals became detached and dentine's structure collapses [33, 34]. In this way, cariogenic biofilm challenge would weaken dentine and generate caries-like lesions. In support of this process, we found that dentine carious lesions that had undergone SDF application had significantly lower log values of [amide I: HPO_4^{2-}] absorbance ratios than did the controls. This finding may indicate that SDF slow down the extension of demineralization in dentine.

Conclusion

Present study has comprehensively showed that SDF possess an anti-microbial activity against cariogenic biofilms of *S. mutans* or *A. naeslundii* formed on dentine surfaces. In addition, SDF slowed down demineralization of dentine. This dual activity could be the reason behind clinical success of SDF.

Acknowledgments

References

1. C. H. Chu, E. C. Lo and H. C. Lin,Effectiveness of silver diamine fluoride and sodium fluoride varnish in arresting dentin caries in Chinese pre-school children.J Dent Res.2002;81;767-70

2. J. C. Llodra, A. Rodriguez, B. Ferrer, et al.,Efficacy of silver diamine fluoride for caries reduction in primary teeth and first permanent molars of schoolchildren: 36-month clinical trial.J Dent Res.2005;84;721-4

3. E. C. Lo, Y. Luo, H. P. Tan, et al.,ART and conventional root restorations in elders after 12 months.J Dent Res.2006;85;929-32

4. C. H. Chu and E. C. Lo,Promoting caries arrest in children with silver diamine fluoride: a review.Oral Health Prev Dent.2008;6;315-21

5. A. Rosenblatt, T. C. Stamford and R. Niederman,Silver diamine fluoride: a caries "silver-fluoride bullet".J Dent Res.2009;88;116-25

6. M. M. Braga, F. M. Mendes, M. S. De Benedetto, et al.,Effect of silver diammine fluoride on incipient caries lesions in erupting permanent first molars: a pilot study.J Dent Child (Chic).2009;76;28-33

7. A. Kawasaki, T. Suge, K. Ishikawa, et al.,Ammonium hexafluorosilicate increased acid resistance of bovine enamel and dentine.J Mater Sci Mater Med.2005;16;461-6

8. C. H. Chu and E. C. Lo,Microhardness of dentine in primary teeth after topical fluoride applications.J Dent.2008;36;387-91

9. G. M. Knight, J. M. McIntyre, G. G. Craig, et al.,Differences between normal and demineralized dentine pretreated with silver fluoride and potassium iodide after an in vitro challenge by Streptococcus mutans.Aust Dent J.2007;52;16-21

10. G. M. Knight, J. M. McIntyre, G. G. Craig, et al.,Inability to form a biofilm of Streptococcus mutans on silver fluoride- and potassium iodide-treated demineralized dentin.Quintessence Int.2009;40;155-61

11. R. G. Quivey,Caries.Oral microbiology and immunology.2006;

12. E. Newbrun,Cariology.1989;

13. H. K. Yip, J. H. Guo and W. H. Wong,Incipient caries lesions on cementum by mono- and co-culture oral bacteria.J Dent.2007;35;377-82

14. J. M. ten Cate and P. P. Duijsters,Alternating demineralization and remineralization of artificial enamel lesions.Caries Res.1982;16;201-10

15. A. Itthagarun, N. M. King, J. S. Wefel, et al.,The effect of fluoridated and non-fluoridated rewetting agents on in vitro recurrent caries.J Dent.2001;29;255-73

16. R. Z. Thomas, J. L. Ruben, J. J. ten Bosch, et al.,Effect of ethylene oxide sterilization on enamel and dentin demineralization in vitro.J Dent.2007;35;547-51

17. M. Shu, L. Wong, J. H. Miller, et al.,Development of multi-species consortia biofilms of oral bacteria as an enamel and root caries model system.Arch Oral Biol.2000;45;27-40

18. P. D. Marsh and B. Nyvad,The oral microflora and biofilms on teeth.Dental Caries The Disease and its Clinical Management.2008;164-187

19. Y. H. Samaranayake, J. Ye, J. Y. Yau, et al.,In vitro method to study antifungal perfusion in Candida biofilms.J Clin Microbiol.2005;43;818-25

20. C. J. Seneviratne, R. W. Wong and L. P. Samaranayake,Potent anti-microbial activity of traditional Chinese medicine herbs against Candida species.Mycoses.2008;51;30-4

21. H. K. Yip, J. Guo and W. H. Wong,Protection offered by root-surface restorative materials against biofilm challenge.J Dent Res.2007;86;431-5

22. R. Yamaga, M. Nishino, S. Yoshida, et al.,Diammine silver fluoride and its clinical application.J Osaka Univ Dent Sch.1972;12;1-20

23. M. Y. Wu, K. Suryanarayanan, W. J. van Ooij, et al.,Using microbial genomics to evaluate the effectiveness of silver to prevent biofilm formation.Water Sci Technol.2007;55;413-9

24. S. R. Brailsford, B. Shah, D. Simons, et al.,The predominant aciduric microflora of root-caries lesions.J Dent Res.2001;80;1828-33

25. Y. Hosoya, S. J. Marshall, L. G. Watanabe, et al.,Microhardness of carious deciduous dentin.Oper Dent.2000;25;81-9

26. L. Angker, N. Nijhof, M. V. Swain, et al.,Influence of hydration and mechanical characterization of carious primary dentine using an ultra-micro indentation system (UMIS).Eur J Oral Sci.2004;112;231-6

27. M. Hossain, Y. Nakamura, Y. Murakami, et al.,A comparative study on compositional changes and Knoop hardness measurement of the cavity floor prepared by Er:YAG laser irradiation and mechanical bur cavity.J Clin Laser Med Surg.2003;21;29-33

28. A. T. Hara, R. L. Karlinsey and D. T. Zero,Dentine remineralization by simulated saliva formulations with different Ca and Pi contents.Caries Res.2008;42;51-6

29. M. Hennequin and Y. Douillard,Effects of citric acid treatment on the Ca, P and Mg contents of human dental roots.J Clin Periodontol.1995;22;550-7

30. Y. Takeuchi, B. Guggenheim, A. Filieri, et al.,Effect of chlorhexidine/thymol and fluoride varnishes on dental biofilm formation in vitro.Eur J Oral Sci.2007;115;468-72

31. W. H. Arnold, S. Konopka, M. S. Kriwalsky, et al.,Morphological analysis and chemical content of natural dentin carious lesion zones.Ann Anat.2003;185;419-24

32. R. Sakoolnamarka, M. F. Burrow, M. Swain, et al.,Microhardness and Ca:P ratio of carious and Carisolv treated caries-affected dentine using an ultra-micro-indentation system and energy dispersive analysis of x-rays--a pilot study.Aust Dent J.2005;50;246-50

33. L. M. Silverstone, N. W. Johnson, J. M. Hardie, et al.,The nature and problem of dental caries in man.Dental caries Aetiology, Pathology and Prevention.1981;10

34. J. M. ten Cate, M. J. Larsen, E. I. F. Pearce, et al.,Chemical interactions between the tooth and oral fluids.Dental Caries The Disease and its Clinical Management.2008;209-230

Table

Table 1 Bacterial count (log CFU) and ratio of dead: live bacteria in two mono-species biofilms (N=8 in each group)
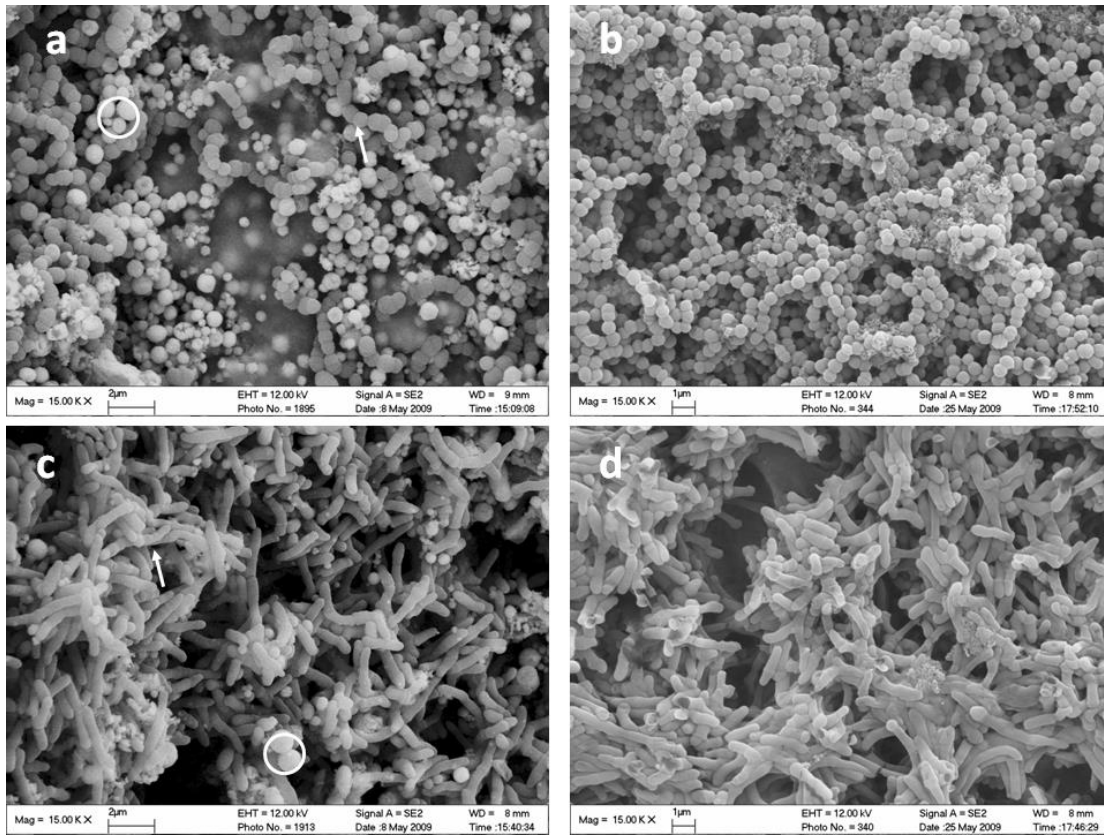
Group	S. mutans		A. naeslundii	
Treatment	log CFU	Dead:live bacteria ratio	log CFU	Dead:live bacteria ratio
SDF	0	5.61±2.42	0	16.01±10.83
Control	6.03±0.18	0.025±0.01	7.00±0.24	0.23±0.09
p value	<0.001	<0.01	<0.001	<0.05

Table 2 Log FTIR intensity ratio [amide I: HPO_4^{2-}] of lesion surfaces after mono-species biofilm challenge (N=8 in each group)

Bacterial species	SDF	Control (7 days after SDF application)	p value
S. mutans	0.57 ± 0.12	1.10 ± 0.28	0.04
A. naeslundii	0.38 ± 0.17	0.93 ± 0.14	0.01

Figure

Figure 1. SEM images of biofilms with and without SDF treatment (×15,000)

a. S. mutans biofilm with SDF; b. *S. mutans* biofilm; c. *A. naeslundii* with SDF;
d. *A. naeslundii* biofilm
Arrow: bacteria; Circle: silver particle

Figure 2 CLSM images of biofilms with and without SDF treatment (×600)
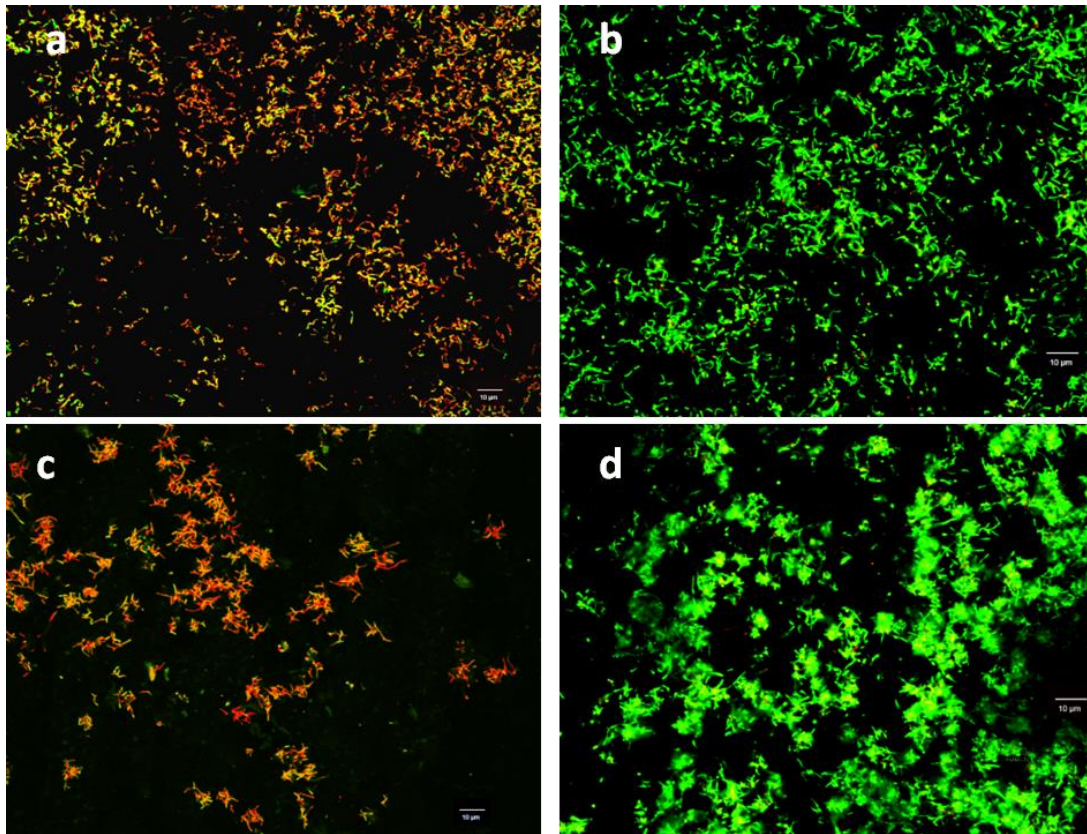
a. *S. mutans* biofilm with SDF; b. *S. mutans* biofilm; c. *A. naeslundii* with SDF;
d. *A. naeslundii* biofilm

Figure 3 Relative microhardness of carious dentine with *S. mutans* biofilm (n=8)
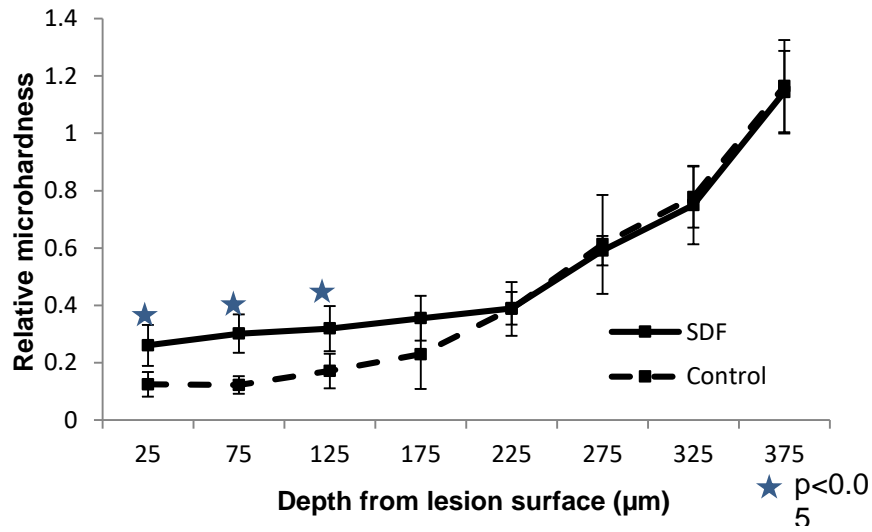
Figure 4 Relative microhardness of carious dentine with *A. naeslundii* biofilm (n=8)
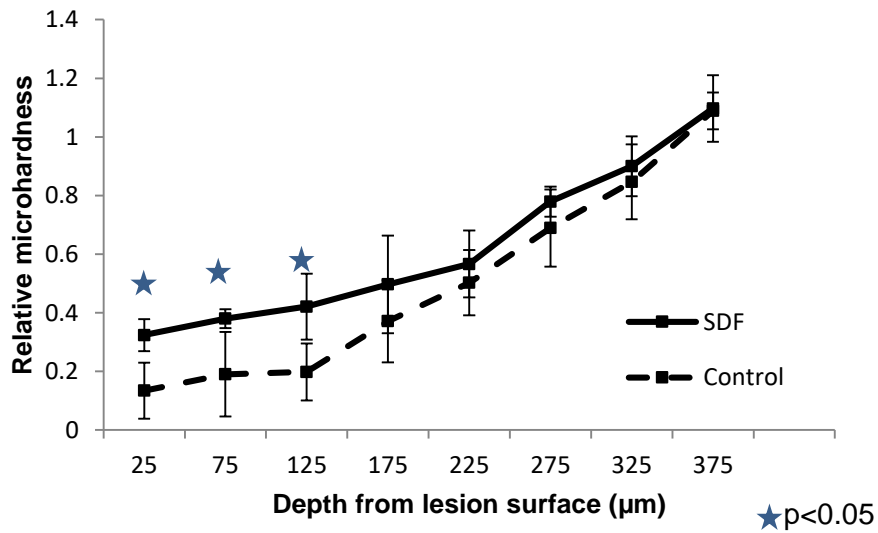
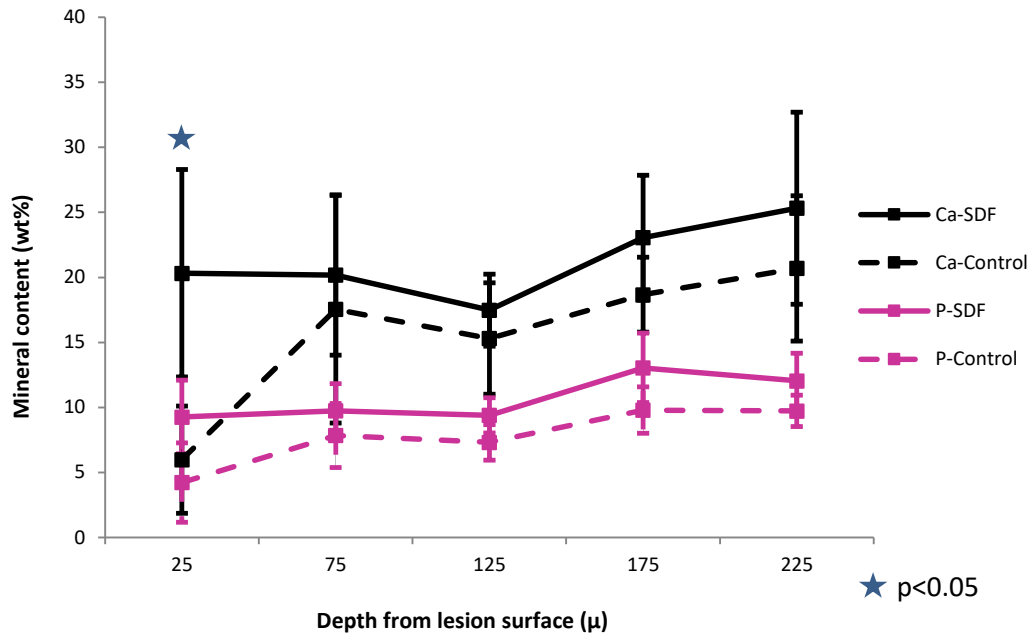Figure 5 Mineral content (wt %) in carious dentine with *S. mutans* biofilm (n=8)

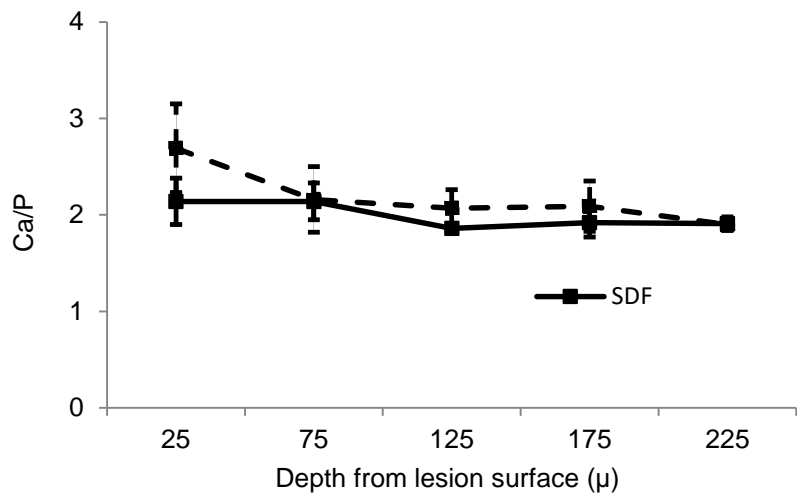Figure 6 Ca/P in carious dentine with *S. mutans* biofilm (n=8)

Figure 7 Mineral content (wt %) and Ca/P in carious dentine with *A. naeslundii* biofilm (n=8)
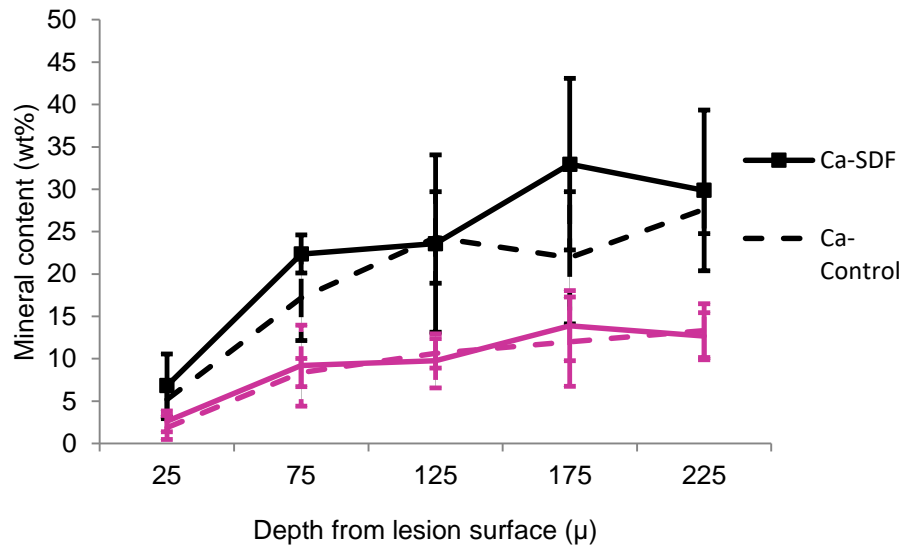

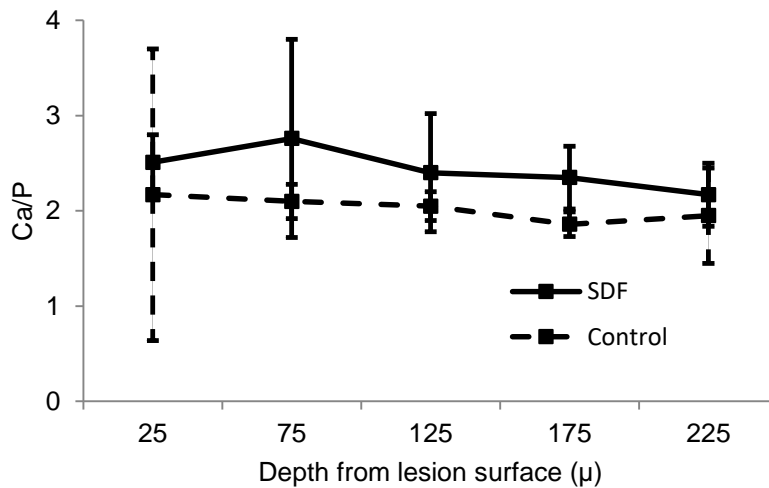Figure 8 Ca/P in carious dentine with *A. naeslundii* biofilm (n=8)

Figure 9 FTIR spectra of sound and chemical demineralised dentine