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

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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 eight 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 were harder after SDF application than after water application ($P < 0.05$), 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 Silver diamine fluoride (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 root surface of permanent teeth in adult[2]. Recent systematic reviews of human clinical trials indicate that silver diamine fluoride is a more effective anticariogenic agent than fluoride alone[3,4]. The mechanism of action of SDF is hypothesised to be its anticariogenic properties[5,6] and its ability to increase enamel surface microhardness and reduce enamel surface mineral loss[7,8]. Neither the mechanisms of action nor their optimisations are well understood. We carried out microbial, chemical and physical measurements of SDF *in vitro*, in an attempt to better understand these parameters. As *S. mutans* is the most predominate bacteria related with caries[9], *A. naeslundii* was highly associated with root caries[10]. Hence, this study investigated the antimicrobial effect of SDF on *S. mutans* and *A. naeslundii* biofilms as well as the mineral content of dentine caries lesions. The outcomes measures are colony-forming unit and dead-to-live ratio of the biofilms, and microhardness, calcium and phosphate content and matrix-to-phosphate ratio of the carious lesions.

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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 $2 \times 2 \times 4$ mm^3 were prepared as described in a previous study[11] and divided into two groups. We assumed the mean lesion depth before and after the 7-day of bacterial demineralisation challenge were 100 and 150 μm and the common standard deviation was 35 μm. The sample size was at least eight in each group with power at 0.80 and $\alpha = 0.05$. 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 and speed up the subsequent 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 h at 23°C[12,13]. The blocks were then sterilised with ethylene oxide (Amsco Eagle 2017 EO steriliser; STERIS, Mentor, USA) for 16 h[14].

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-h broth cultures in basal medium supplemented with 5% glucose (BMG medium) at 37°C under anaerobic conditions[15]. 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 300 μL aliquot of each bacteria was inoculated on each demineralised dentine block (eight blocks for each bacterium) sitting in 1 mL 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 days also allowed maturation of the biofilm[16]. The medium was refreshed daily without disturbing the specimen surface. After 7 days' incubation, eight 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 ± 0.07 mg (or 8.8 ± 2.8 μg fluoride), as estimated by calculating the difference of the gravimetric microbrush before and after application. The other eight 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 75 rpm inside the anaerobic chamber for 7 days at 37°C.

Study of biofilm characteristics—microbiota

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 homogenised biofilm samples in 1% PBS were plated in duplicate with a spiral plater (Autoplate 4000; Spiral Biotech Inc., Norwood, MA, USA) onto horse blood agar (Defib Horse Blood; Hemostat Laboratories, Dixon, CA, USA). As fluoride ions might react with SiO_2 and cause inaccurate pH reading by a pH sensor, 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 (eight categories).

Scanning electron microsocpy (SEM) was used to examine the topographical features of the biofilm. In preparation for SEM[17], 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 labelled *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 labelling[18]. Thereafter, four 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, Bethesda, MD, USA). The red-to-green ratio was calculated to indicate the ratio of dead-to-live bacteria on the antimicrobial effect of the therapeutic agent.

Study of hard tissue characteristics—physical assessment

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) 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 150–200 μm apart, and Knoop microhardness measurements were made using computer software (Leica QGo-Applet Runner; Ernst Leitz). 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].

Study of hard tissue characteristics—chemical assessments

The mineral content as levels of Ca and P of dentine lesions was analysed 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 analysed with a Bio-Rad FTIR UMA-500 machine (Bio-Rad Laboratories, Hercules, CA, USA), with infrared radiation ranging from 650 to 4000/cm 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 to the area of absorbance of the HPO_4^{2-} peak between 900 and 1200/cm. 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 because of the carious activity of the biofilm[19].

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, IL, USA). The cut-off level of significance was taken as 5% for all analyses.

Results

Biofilm characteristics—microbiota

The results of this study demonstrated that SDF significantly reduced the CFU counts of *S. mutans* and *A. naeslundii*. 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 higher than those in the control groups.

In the SDF treatment groups, round particles of about 0.5–1 μm that were visible in both *S. mutans* groups and *A. naeslundii* in SEM images (Fig. 1a,c 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 (Fig. 1). *Actinomyces naeslundii* biofilms were seen as typical 'Y'-shaped branched networks (Fig. 1). Most of the bacteria present in the biofilm fluoresced green in control group indicate that the bacteria were mostly alive in control. The dead-to-live ratios from CLSM images (Fig. 2), which

Table 1. Bacterial count (log Colony-Forming Unit (CFU)) and ratio of dead-to-live bacteria in two mono-species biofilms (*N* = 8 in each group).

Group	*Streptococcus mutans*		*Actinomyces naeslundii*	
Treatment	log CFU	Dead:live bacteria ratio	log CFU	Dead:live bacteria ratio
Control	6.03 ± 0.18	0.025 ± 0.01	7.00 ± 0.24	0.23 ± 0.09
SDF	0	5.61 ± 2.42	0	16.01 ± 10.83
P value	<0.001	0.01	<0.001	0.04

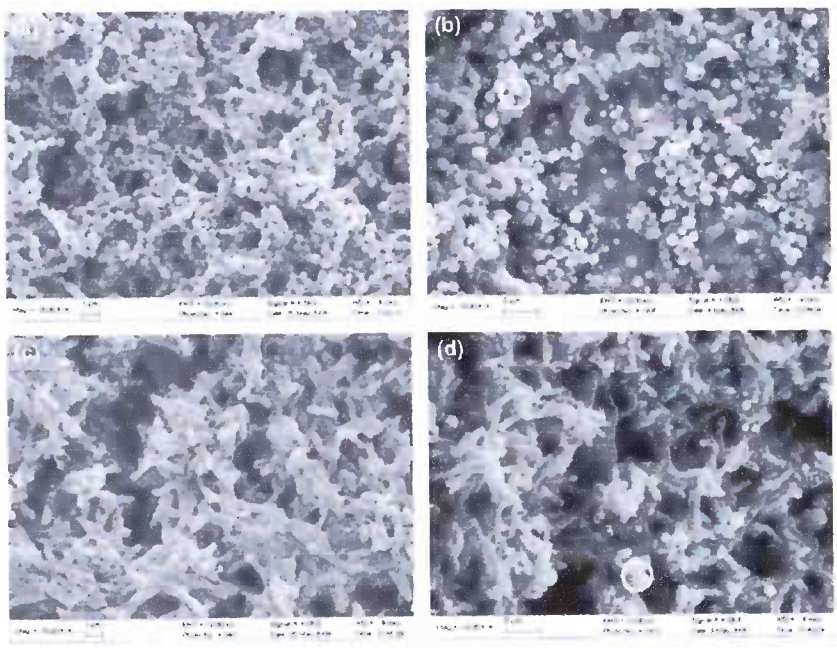

Fig. 1. Scanning electron microscopy images of biofilms in control and silver diamine fluoride (SDF) groups (×15,000). (a) *Streptococcus mutans* biofilm; (b) *S. mutans* biofilm with SDF; (c) *Actinomyces naeslundii* biofilm; (d) *A. naeslundii* with SDF; Circle: silver particle conformed by energy-dispersive x-ray spectroscopy.

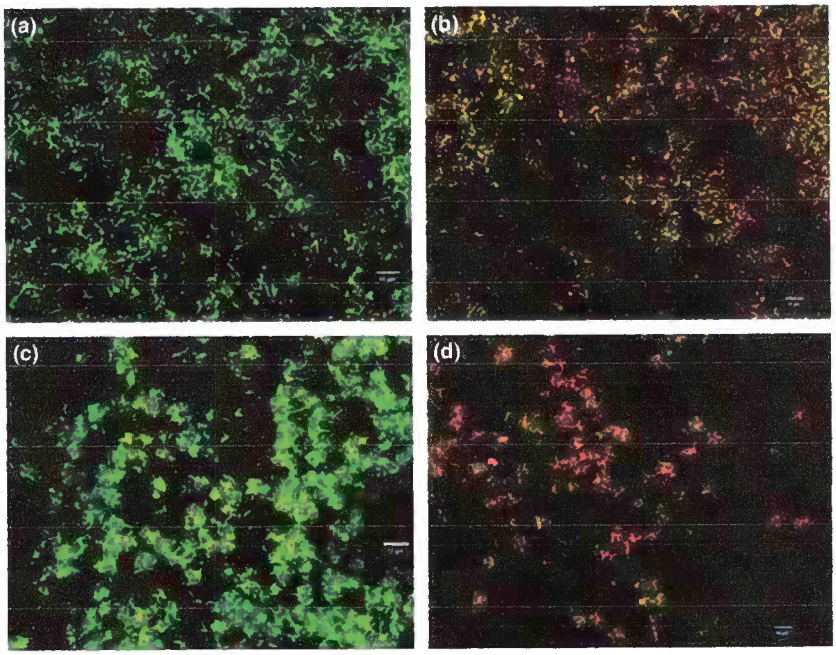

Fig. 2. Confocal laser scanning microscopy images of biofilms in control and silver diamine fluoride (SDF) groups (×600). (a) *Streptococcus mutans* biofilm; (b) *S. mutans* biofilm with SDF; (c) *Actinomyces naeslundii* biofilm; (d) *A. naeslundii* with SDF; Red—dead bacteria, green—live bacteria.

indicate strength of antimicrobial 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 and 5.0 and the value increased to 6.0–6.5 with SDF treatment.

Hard tissue characteristics—physical assessment

The results of this study demonstrated that SDF significantly reduced the deterioration in microhardness of the dentine caries lesion. The outer surface up to 125 μm of the dentine carious lesions because of *S. mutans* and *A. naeslundii* in the SDF subgroup was significantly harder than in the control subgroup ($P < 0.05$; Fig. 3).

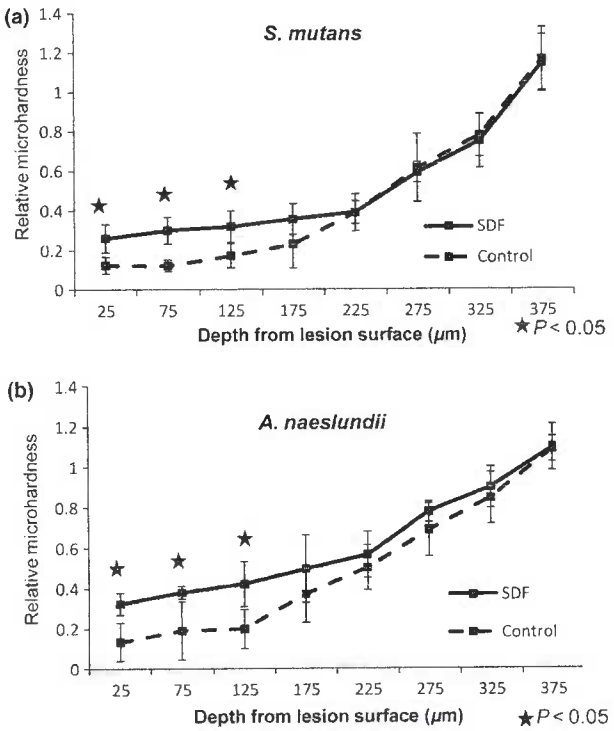

Fig. 3. Relative microhardness of carious dentine with biofilms ($n = 8$). (a) Relative microhardness of carious dentine with *Streptococcus mutans* biofilm. (b) Relative microhardness of carious dentine with *Actinomyces naeslundii* biofilm.

Hard tissue characteristics—chemical assessments

The results of this study demonstrated that SDF significantly reduced the mineral content on the surface of the dentine caries lesion–bearing *S. mutans* biofilms. 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, $P < 0.05$; Fig. 4). No significant difference of the Ca/P ratio in all measured depths from the surface (25–225 μm)

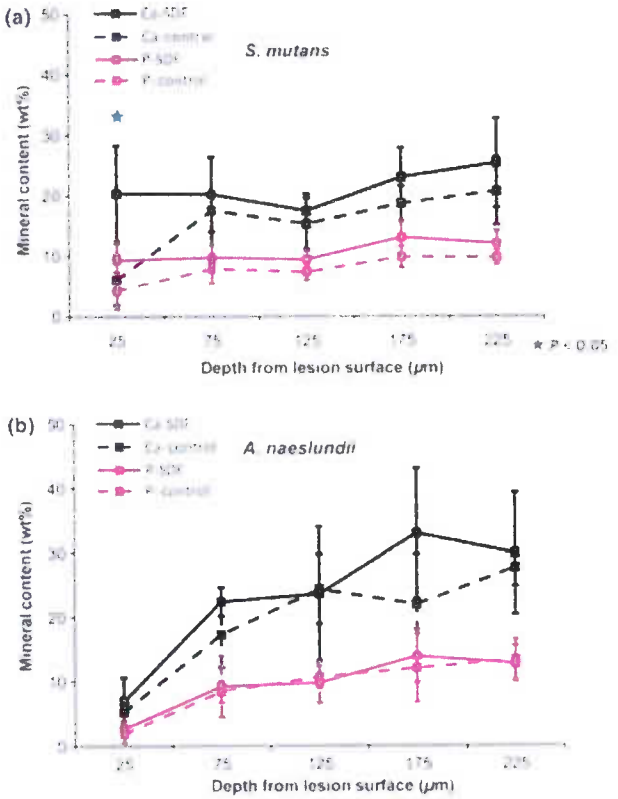

Fig. 4. Mineral content (wt %) in carious dentine with biofilms (n = 8). (a) Mineral content (wt %) in carious dentine with *S. mutans* biofilm. (b) Mineral content (wt %) in carious dentine with *Actinomyces naeslundii* biofilm.

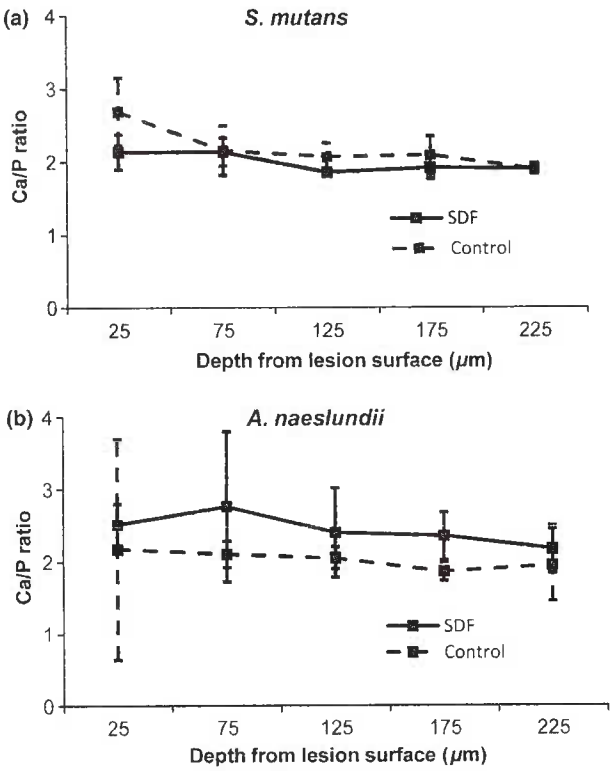

Fig. 5. Ca/P ratio in carious dentine with biofilms (n = 8). (a) Ca/P ratio in carious dentine with *Streptococcus mutans* biofilm. (b) Ca/P ratio in carious dentine with *Actinomyces naeslundii* biofilm.

was found however (Fig. 4). 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 (Fig. 5).

The results of this study demonstrated that SDF significantly reduced the demineralisation of the dentine caries lesion. The FTIR spectra of sound dentine and artificial caries lesion were shown in Fig. 6. For the dentine, the absorbance for amide I occurred at 1585 to 1720/cm and that for HPO_4^{2-} was from 900 to 1200/cm. The intensity of the phosphate band was strong in sound dentine but was weak in demineralised dentine; amide I band showed a little bit higher in demineralised dentine than that in sound dentine. Inter-specimen 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 2. In

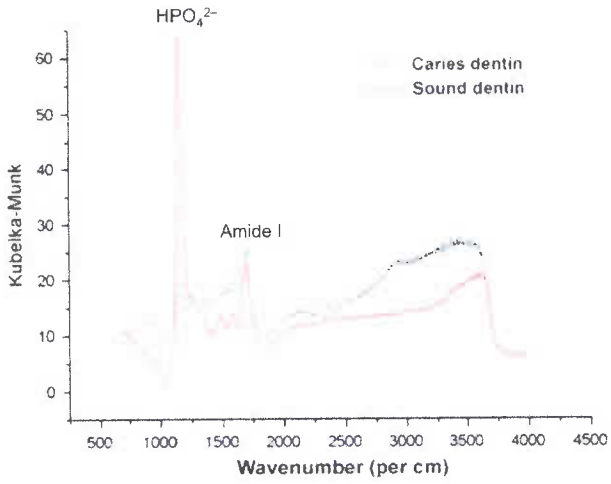

Fig. 6. An example of Fourier transform infrared (FTIR) spectroscopy spectra to illustrate the Amide I and HPO_4^{2-} absorption of sound dentine and carious dentine. Amide I-peak between 1585 and 1720/cm; HPO_4^{2-} -peak between 900 and 1200/cm.

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.

Table 2. Fourier transform infrared spectroscopy intensity ratio Log [amide I: HPO_4^{2-} of lesion surfaces after mono-species biofilm challenge ($N = 8$ in each group).

Bacterial species	Control	SDF	*P* value
Streptococcus mutans	1.10 ± 0.28	0.57 ± 0.12	0.04
Actinomyces naeslundii	0.93 ± 0.14	0.38 ± 0.17	0.01

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[20]. Wu *et al.*[21] demonstrated silver has antibacteria 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[4].

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*). *Streptococcus 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[22]. Mono-species biofilms in a microplate system, however, 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[8,23,24]. 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[25–27], 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[25–27]. We also found that Ca and P weight percentages in the outer 25 μm of dentine carious lesions because of *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[28].

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[29,30]. 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 increased by cariogenic biofilm challenge[27]. 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[28]. 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 mineralised dentine. The result of this study confirms the result reported by an early study[29]. 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[28]. 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[31,32]. 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 demineralisation in dentine.

Conclusion

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

What this paper adds

- This study adds information on the mechanisim of SDF on cariogenic biofilm and caries.

Why this paper is important for paediatric dentists

- SDF is a safe, simple and effective agent in caries management among children. This study provides basic knowledge of SDF effect on caries.

Acknowledgements

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