INTRODUCTION

Dental caries is the most prevalent chronic disease that involves infection by cariogenic bacteria and degressive destruction of tooth structures. This disease process initiates with the adhesion, proliferation of oral bacteria, eventual plaque formation and acid secretion subsequently causing demineralization of dental hard tissues1-3). Similar process of caries formation is being recognized on the exposed root surfaces which end up with formation of root caries4-6). It is an increasing clinical manifestation with the growth in the dentate of elderly population during the last two decades7). Among the limited number of treatment modalities application of silver diamine fluoride (SDF; Ag[\(\text{NH}_3\)\(_2\)]\(_2\)\(\text{F}\)) is becoming increasingly popular in terms of arresting dental caries8,9). In a recent meta-analysis study it was shown that annual applications of SDF regimens were more effective than the control in preventing root caries10). More recently, a 10 fold dilution of the original 38% SDF solution (3.8%-SDF) has also been developed for intracanal irrigation and is being used with notable clinical success rate11). Studies on biofilm models reported that 3.8%-SDF application in human root canal effectively inhibited the growth of \textit{E. faecalis} in the biofilm12,13).

Regrettably however, the topical application of SDF causes a staining of organic components as found in pellicle and active caries14), due to the reaction of free silver ions (Ag+) with organic substances; seen in dentin too15). A new approach has been developed to overcome this problem by applying a saturated solution of potassium iodide (KI) immediately after SDF application16,17).

\textit{Streptococcus mutans} (\textit{S. mutans}) is considered the primary etiologic oral pathogen of dental caries, secondary caries and even root caries is being widely used for \textit{in vitro} studies all around the world5,18,19). To date, none of the above three SDF preparations have been tested against biofilm forming \textit{S. mutans} in an \textit{in vitro} platform of RD. A computer controlled oral biofilm reactor (OBR) is being used for \textit{in vitro} formation of cariogenic biofilms to contribute in the research projects for prevention of caries and/or secondary caries and development of new dental materials20,21). Accordingly, this study was designed to investigate the effects of three preparations of SDFs on \textit{S. mutans} biofilm formation is almost similar, although not equivalent to 38%-SDF.

**MATERIALS AND METHODS**

**Specimen preparation**

Square shaped RD blocks (5x5x2 mm\(^3\)) were prepared from bovine incisors using a low-speed diamond saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA) under copious water coolant. The surfaces were ground flat exposing the dentin using 2000-grit silicon carbide papers and further polished using 0.25 \(\mu\)m Diamond
Paste (DP; Paste P 1/4μm, Struers, Ballerup, Denmark) then ultra-sonicated in deionized water (DiW; Millipore, Billerica, MA, USA).

**Study groups, SDF reagents and application**

For each experiment twenty blocks were prepared, sixteen were used for each biofilm study and rest were used other investigations as described below.

The dentin specimens were randomly divided into four groups as follows:

Control; a negative control group was allotted without application of any material.

Three SDF preparations used in study as experimental groups are

- **38%-SDF:** 38% solution of silver diamine fluoride (Saforide, Bee Brand Medico Dental, Osaka, Japan).
- **3.8%-SDF:** 3.8% solution of silver diamine fluoride (Saforide RC, Bee Brand Medico Dental).
- **SDF+KI:** Unit 1; 0.05-mL solution of 30–35% silver diamine fluoride. Unit 2; 0.10-mL of KI solution contains potassium, iodine and methacrylates (Riva Star; SDI Dental, Bayswater, Australia).

Materials were applied according to manufacturer instructions. For Control: no material was applied. For 38%-SDF: material was applied to the RD specimen surface and agitated using a micro-brush for 10 s, left for 3 min from application and rinsed with water using three way syringe vertically for 10 s. For 3.8%-SDF: material was applied to the RD specimen surface and agitated using a micro-brush for 10 s, left for 3 min from application and rinsed with water using three way syringe vertically for 10 s. For SDF+KI: A layer of SDF solution from the silver capsule (Unit 1) was applied on the RD specimen surface and agitated using a micro-brush for 10 s. Then a generous amount of KI solution from green capsule (Unit 2) was immediately applied on the SDF-treated surface, until the formed creamy white color turned clear, left for 3 min from SDF application and rinsed as described above.

**Bacterial isolate and growth media**

A laboratory strain of cariogenic bacteria, *Streptococcus mutans* MT8148 (*S. mutans*) was used in this study. Bacteria cultures were established in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA). After 18 h, bacterial cells washed 3 times with phosphate-buffered saline (PBS), and suspended in PBS (OD 490=1.05).

For growth cultivation of *S. mutans* biofilm, a solution of heart infusion broth (Becton Dickinson) with sucrose (1.0% final concentration; HI-sucrose) was utilized.

**Biofilm formation in the OBR**

The method has been described in articles before. In brief, *S. mutans* suspension was used to form biofilms on the specimen surfaces that had been placed inside the water jacket-encircled chamber of an OBR as shown in Fig. 1. Sixteen blocks were positioned on two specimen holders in OBR chambers around a flat-bulb pH electrode. The chamber itself served as an incubator with reduced oxygen level a 37°C internal temperature. The suspension of *S. mutans*, HI-sucrose, and PBS were pumped into the chambers to continuously drop onto the center of the specimen holder. All of these solutions formed a liquid dome on the holder which was stirred by the falling drops. As a continuous process the dome reformed with fresh solutions. Biofilms were formed on the RD specimens inside the chamber of the OBR.

**Quantitative assessment of the biofilms**

To obtain a measurable amount of biofilms on each group of specimen OBR was operated for 20 h. After carefully removing the specimens with 20-h biofilms from OBR were lightly rinsed using PBS to remove the planktonic bacteria and loosely attached biofilms. The bacterial cells and water-insoluble glucan (WIG) matrix were then separated using 0.5 mol/L sodium hydroxide (NaOH) solutions (NaOH). Centrifuged (10,000 g for 5 min) bacterial pellets were separately resuspended in 1 mL of PBS after transferring the dissolved WIG solutions.

Each bacterial suspension was transferred into 96-well flat-bottom microplates to quantify the bacteria by turbidimetric analysis (OD 490) using a spectrophotometer (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA). The amount of dissolved WIG was measured by the phenol-H₂SO₄ method. The WIG solution (250 μL) from each sample was disintegrated.
with phenol-H$_2$SO$_4$, and 200 μL of each was used to estimate the amounts of WIG (μL /g/mL) using the same spectrometer. All experiments were repeated three times for reproducibility.

**Biofilm thickness measurement**

To verify any effect on biofilm growth the thickness of 20 h biofilm was measured using a non-invasive swept-source optical coherence tomography (SS-OCT; IVS-2000, Santec, Komaki, Japan). The SS-OCT probe was set on a holder at a fixed distance from specimen with the scanning beam oriented perpendicular with respect to the RD surfaces. Minimum of 10 cross-sectional images were obtained scanning the biofilm attached on each RD specimen surface after blot-socking extra PBS. From the 2-dimentional images from each group thickness of the biofilms were calculated counting the pixels and converting into μm.

**Fluorescence microscopy of the biofilms**

A LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Carlsbad, CA, USA) was used to observe the bactericidal effect of the SDFs after application on RD surface. Specimens with S. mutans biofilms formed in 2 h using the same procedures mentioned above were lightly washed with PBS and stained with 0.2 μL BacLight stain (a mixture of SYTO 9 and propidium iodide (PI)). Viability of S. mutans cells either free or in biofilms clusters, detectable at this early stage of biofilm formation, were then inspected using both Longpass and Bandpass emissions of a fluorescence microscope (CKX41, Olympus, Tokyo, Japan). Live and dead bacteria were visualized by green signals from SYTO 9 and red signals from PI respectively.

**Morphological observations**

Specimens from all four groups with 2-h biofilms were observed using a SEM (JSM-IT100 InTouchScope, JEOL, Tokyo, Japan) as this early stage of biofilms formation presents with newly developed small biofilm clusters, fresh bacterial cells, free spaces of RD surface and even some SDF precipitates. Each specimen surface was almost entirely inspected several times and images were taken at various magnifications with S. mutans initial biofilms that were formed in 2 h. The specimens were removed from the OBR chamber, rinsed with PBS and fixed with 2.5% glutaraldehyde. They were then rinsed with PBS and DiW, followed by desiccation and sputter gold coating before SEM observation.

**Statistical analysis**

Statistical analyses were performed with SPSS 15.0 for Windows. Amounts of bacteria and WIG were analyzed using one way ANOVA and Tukey’s HSD. Comparison of biofilm thickness performed by means of the Mann–Whitney-U test.

**RESULTS**

**Quantity of biofilms**

The amounts of biofilm formation were reasonably different among the four groups (Fig. 2). Significantly

![Fig. 2](image-url) Represents the amounts of S. mutans biofilm formed on specimen surfaces in 20 h inside OBR; amounts of bacteria (left) and amounts of WIG (right). Average±standard deviations, different letters highlight statistically significant differences between groups (p<0.05).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Thickness of attached biofilms expressed in μm; different letters highlight statistically significant differences between groups (p&lt;0.05)</th>
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<tbody>
<tr>
<td>Control (Av±SD)</td>
<td>38%-SDF (Av±SD)</td>
</tr>
<tr>
<td>Control (Av±SD)</td>
<td>38%-SDF (Av±SD)</td>
</tr>
<tr>
<td>944±365$^a$</td>
<td>206±90$^a$</td>
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smaller amounts of bacteria and glucan were calculated in all three SDF groups compared to Control. Expectedly, 38%-SDF had significantly least amounts of bacteria and glucan. There were no significant differences in the amounts of bacteria/mm² between 3.8%-SDF and SDF+KI. However, 3.8%-SDF presented with small amount of glucan which was significantly less than that of SDF+KI.

**Thickness of attached biofilms**
Application of SDFs on RD surfaces inhibited growth of the biofilms as result the thickness of 20 h biofilms did not increase as same as Control (Table 1). Feasibly, 38%-SDF had significantly least thick biofilm. There were no significant differences in the thickness between 3.8%-SDF and SDF+KI, but had significantly thinner biofilms than Control.

**Viability of bacteria**
An interesting trend of the fluorescence patterns could be detected among the groups (Fig. 3). As obvious, most of the bacteria were alive in Control — apparently were active as bright green signals were observed for a longer period. Conversely, red clusters were all over the surface on 38%-SDF specimen, rarely green bacteria could be seen for a few seconds. While, in 3.8%-SDF and SDF+KI smaller amounts of bacteria and glucan were calculated in all three SDF groups compared to Control. Expectedly, 38%-SDF had significantly least amounts of bacteria and glucan. There were no significant differences in the amounts of bacteria/mm² between 3.8%-SDF and SDF+KI. However, 3.8%-SDF presented with small amount of glucan which was significantly less than that of SDF+KI.

**Fig. 3** Fluorescence photomicrography of 2-h *S. mutans* biofilms stained with a BacLight bacterial viability kit (Longpass emission). Biofilm with live cells fluorescing green. Almost all *S. mutans* cells are live (green) and actively forming biofilm in Control. Almost all *S. mutans* cells are dead (red) in 38%-SDF. Dead or dying *S. mutans* cells are visible but live are more in 3.8%-SDF. More live *S. mutans* cells are visible in SDF+KI, dead cell are also seen.

**Fig. 4** Typical SEM images of the root-dentin surfaces from each group; Control, 38%-SDF, 3.8%-SDF and SDF+KI after 2 h of biofilm formation.

a: Images are of ×2k magnification (black arrows show biofilm clusters); on Control specimen a large sized biofilm cluster is seen growing in all dimensions, largest of all. On 38%-SDF, relatively smaller sized biofilm clusters are seen as plenty of Ag-particles remain spread all over the surface and dentinal tubules (DTs) are partially or completely remain obturated by the material. On 3.8%-SDF, biofilms are being formed mainly on the intertubular spaces where Ag-particles are less and DTs remained partially obturated by the material. On SDF+KI, larger biofilm clusters compared to other two SDFs are seen, few material particles are seen spread scattered and DTs are open.

b: Images are of ×10k; on Control specimen *S. mutans* appear to be morphologically normal, some are extending adhesins (dotted arrows) to get adhered to dentin, some are proliferating and synthesizing glucan. On 38%-SDF, normal growth of bacterial cells is impaired — size remain small, slander in shape and appear to have taken black stain as they are seen attached with the Ag particles (white circles). On 3.8%-SDF, change in morphology and color of the bacteria can be detected but not as same as 38%-SDF. On SDF+KI, little change in morphology and color was observed, but normal biofilm formation can also be detected.
groups bactericidal effect was not instant, required a few seconds before could be confirmed as dead bacteria; still, proportionally large number of biofilm clusters emitted green signals.

**SEM observation**

Presence of 2-h initial biofilms could be detected on all specimen surfaces (Fig. 4). Normal looking *S. mutans* chains and growing biofilms with glucan like extracellular polymeric substances (EPS) was observed in Control group. However, free or embedded bacteria within the EPS appeared different in three SDF groups; changes in size, shape and color were evident both in the biofilms and in bacterial cells that remained attached with material particles, mainly in 38%-SDF. A large volume of SDF precipitates remained on 38%-SDF, on SDF+KI were very few and on 3.8%-SDF was in between (Fig. 4a). Morphological deformation of bacteria and darkening was extensively severe in 38%-SDF group, a few ruptured cells were also seen. Also, attachments of *S. mutans* to the dentin surface via their adhesins were seen in Control that could not be detected on any of the SDF specimens (Fig. 4b).

On reexamining SEM images, most of DTs found remained closed with silver derived crystals in 38%-SDF group preventing bacterial intrusions into DTs, which was a common picture for the Control. Fresh looking *S. mutans* were proliferating making chains and trying to intruding into the DTs on the latter. No *S. mutans* chains could be detected on 38%-SDF specimen. SDF+KI had less bacterial intrusion than Control and 3.8%-SDF had fewest. Interestingly, 3.8%-SDF material particle remained attached on the peritubular walls, keeping a smaller opening at the middle.

**DISCUSSION**

Overwhelming bactericidal effect of 38%-SDF as the hallmark property has been revealed in this study. This time it was shown on the biofilm forming *S. mutans* in an environment notably favorable for the bacteria not only to survive, but even to produce caries on RD surface. It is obvious, because 38%-SDF solution contains two powerful antibacterial metals at extremely high concentrations; 255,000 ppm of Ag and 44,800 ppm of F[28].

In this study, the amount of bacteria, the amount of WIG and the biofilm thickness after 20 h were not absolutely identical for all three SDF reagents; 38%-SDF evidently inhibited bacterial cell growth and equivalently reduced WIG synthesis by infiltrating into the growing biofilms —however though, *S. mutans* desperately continued to form little amount of biofilms. In 3.8%-SDF and SDF+KI groups biofilms continued to form, but there was clear evidence of slowing down that growth in terms of thickness too. Interestingly, 3.8%-SDF had significantly less amount WIG than on SDF+KI, although amounts of bacteria were almost same. WIG as the EPS of *S. mutans*, synthesized using glucosyltransferases from sucrose which are encoded by *gtfB*, *gtfC* and *gtfD* genes, promote adhesion to tooth surfaces[26,27]. Another study found that silver ion inhibited both glucosyl- and fructosyl-transferase activities during synthesis of sucrose-induced *S. mutans* polysaccharides[28]. A similar phenomenon might have occurred in case of SDF+KI, because it lacked in the proportion of Ag from the early stage of biofilm formation. However, as proportion of F was significantly more in SDF+KI than 3.8%-SDF group, that might have made up the shortness of Ag and may have worked as co-inhibitors to reduce the proliferation of *S. mutans*[29].

All three SDF reagents used in this study are delivered as liquid chemicals dissolved in water which precipitates on RD instantly and may have released Ag+ ions as reported before[30]. Also, studies demonstrated that Ag+ ions can electrostatically bind to the anionic portions of the membranes and blocks electron transport system, interact with life-sustaining enzymes and inhibit the movement of the organism or cause the membrane to leak or rupture[31,32]. Seemingly, that scenario happened on biofilm forming *S. mutans* and was practically observed in this study —the bacteria completely lost their original morphology, color darkened as Ag particles remained attached with them. Most likely, the bacteria eventually died due to oxidative stress[32], even their cells ruptured when SDF dose was too high in case of 38%-SDF mainly (0.12 μmol/mL>MIC) —although normal *S. mutans* cell-body is enveloped with thick cell wall around their cell membrane. A sign of photo-canalization was also observed during FM in case of 3.8%-SDF in particular. Furthermore, cell surface proteins such as the cell wall-anchored adhesin are important for *S. mutans* adherence to tooth surfaces[34]. Apparently, Ag+ ions inhibited extension of adhesins which primarily caused weak adherence of *S. mutans* on specimens of all SDF groups in this study.

SDF particles densely precipitated at the orifices of DTs, seemingly, attached with peritubular tissues including collagens in all experimental groups. Perhaps, chemical interaction with dentin structure was also there[35].

As revealed in this study, presence of enough SDF-derived precipitates on the root surface even in an environment of 2 h uninterrupted biofilm growing condition resulted in inhibition of bacterial adhesion, strong bactericidal effect, growth inhibition of biofilms and occlusion of DTs as well, mainly by the 38%-SDF would serve as a mechanism based evidence in favor of dentin hypersensitivity suppression therapy too.

Bactericidal effect of 3.8%-SDF on biofilm forming *S. mutans* was significant enough and potentially inhibited biofilm growth; even it has only 10% of original SDF regimen —besides, it did not stain dentin as severely as 38%-SDF. Unfortunately however, effects of SDF+KI were not up to the expectations even it primarily (Unit 1) contains 30–35% SDF[14,35]. The distribution of silver-derived crystals on the RD surfaces was different from 38%-SDF and 3.8%-SDF which was noticed after application of KI before biofilm attack. While applying KI after SDF, it was realized that Ag+ ions readily reacts with free iodine producing AgI complexes. Furthermore,
Ag particles get washed away rather easily after KI application. As a result, its effect on *S. mutans* biofilms got reduced significantly than 38%-SDF and even from 3.8%-SDF when amount of WIG was compared.

Intrusion of bacterial into the DTs in 3.8%-SDF and SDF+KI groups might open the risk of developing new caries; especially, in SDF+KI applied cases. But compared to the Control, *S. mutans* would not find it easy to enter into the DTs in 3.8%-SDF and SDF+KI groups because the orifices of DTs were at least partially occluded. It is considered that application of 3.8%-SDF is effective enough to prevent root caries as it presented with notable amount of material remaining on the surface even after 2 h of strong biofilm attack and also inhibited biofilm growth. For SDF+KI, although a major part of the silver particles was washed out by the flow of acid during biofilm formation it did inhibit growth of *S. mutans* biofilms.

To have the answer for ‘what to choose to win the combat’ a study like this would be beneficial keeping in mind how to minimize adverse-effects including discoloration of dental hard tissues and fluoride toxicity. It is undoubtedly a significant clinical issue for many patients, although such silver therapy is simple to use and cost effective for caries management, in particular it would benefit specific populations such as patients in developing regions of the world with limited resources, or the frail elderly or institutionalized patients with limited access to dental care.

Attachment of silver particles on biofilm forming *S. mutans* caused discoloration, growth-retardation and rapture of the bacterial cells and the growing biofilms after topical application of 38%-SDF on RD is demonstrated for the first time in this study. It is nevertheless noteworthy that 3.8%-SDF is effective enough and SDF+KI also seem to have moderate effect in inhibiting *S. mutans* biofilm formation on RD surface. However, the latter two may show improved impact in modified conditions —further investigations are required.

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