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Arresting simulated dentine caries with adjunctive application of silver nitrate solution and sodium fluoride varnish: an *in vitro* study

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Purpose: The aim of this *in vitro* study was to assess the ability of silver nitrate solution, followed by sodium fluoride varnish, to arrest caries. **Methods:** Dentine slices were prepared and demineralised. Each slice was cut into three specimens for three groups (SF, SDF and W). Specimens of the SF group received topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish. The SDF group received topical application of 38% silver diamine fluoride solution (positive control). Specimens of the W group received deionised water (negative control). All specimens were subjected to pH cycling for 8 days. Dentine surface morphology, crystal characteristics, carious lesion depth and collagen matrix degradation were evaluated by scanning electron microscopy, X-ray diffraction, X-ray microtomography and spectrophotometry with a hydroxyproline assay. **Results:** Scanning electron microscopy showed that dentine collagen was exposed in group W, but not in groups SF and SDF, while clusters of granular spherical grains were formed in groups SF and SDF. The mean lesion depths (±standard deviation) of groups SF, SDF and W were 128 ± 19, 135 ± 24 and 258 ± 53 μm, respectively (SF, SDF < W; $P < 0.001$). The X-ray diffraction analysis indicated that silver chloride was formed in groups SF and SDF. The concentration of hydroxyproline released from the dentine matrix was significantly lower in groups SF and SDF than in group W ($P < 0.05$). **Clinical significance:** The results of this *in vitro* study indicate that the use of silver nitrate solution and sodium fluoride varnish is effective in inhibiting dentine demineralisation and dentine collagen degradation.

Key words: *Silver nitrate, sodium fluoride, silver diamine fluoride, dentine collagen*

INTRODUCTION

Silver nitrate ($AgNO_3$), an antimicrobial compound, has been used to treat dental caries for more than 100 years[1,2]. However, $AgNO_3$ has not been advocated for use in caries control in the past few decades. Duffin[1] proposed a caries-control protocol in which 25% $AgNO_3$ followed by 5% sodium fluoride (NaF) varnish was applied directly to cavitated carious lesions. He called this protocol 'The medical management of caries with $AgNO_3$' – it is simple and can be readily used in children. The application of $AgNO_3$ followed by fluoride varnish has the advantage that the varnish acts as a protective layer to keep the $AgNO_3$, which is antimicrobial, from being washed away by saliva[3]. Moreover, fluoride could promote caries remineralisation. The protocol is simple and noninvasive. Duffin[1] reported that 98% of the carious lesions of his patients remained arrested for up to 4 years after treatment when using this caries-control protocol. However, no laboratory study on the effect of $AgNO_3$ plus fluoride varnish on caries was found in the literature, and the mechanism of this proposed protocol is unknown. Progression of dentine carious lesions involves both demineralisation and degradation of the organic matrix within the dentine[4]. Once dentine is demineralised, the organic matrix, which is composed of 90% fibrillar type I collagen and 10% non-collagenous proteins, is exposed. The remineralisation process should be regulated by interactions of minerals with the collagen matrix[5,6]. To understand the action of $AgNO_3$ solution and NaF varnish on caries, this study was performed with the objective of investigating the chemical and histological changes in simulated dentine carious lesions subjected to pH cycling after the application of 25% $AgNO_3$ solution and 5% NaF varnish compared with both positive and negative controls.

MATERIALS AND METHODS

Preparing specimens with simulated dentine caries

This study received approval from the Institutional Review Board (the University of Hong Kong) under process number IRB UW14-530. This study was conducted in full accordance with the Declaration of Helsinki of the World Medical Association. All participants received dental treatment at the Faculty of Dentistry of the University of Hong Kong and provided written informed consent. The written consents were obtained from the parents/guardians of the teenagers who were under 18 years of age. The consent procedure was approved by the Institutional Review Board of the University of Hong Kong. The protocol of this study is shown *Figure 1*. Sound third molars were collected from patients who required extraction of such teeth, after gaining the patients' consent. The teeth collected were kept in distilled water at 4 °C.

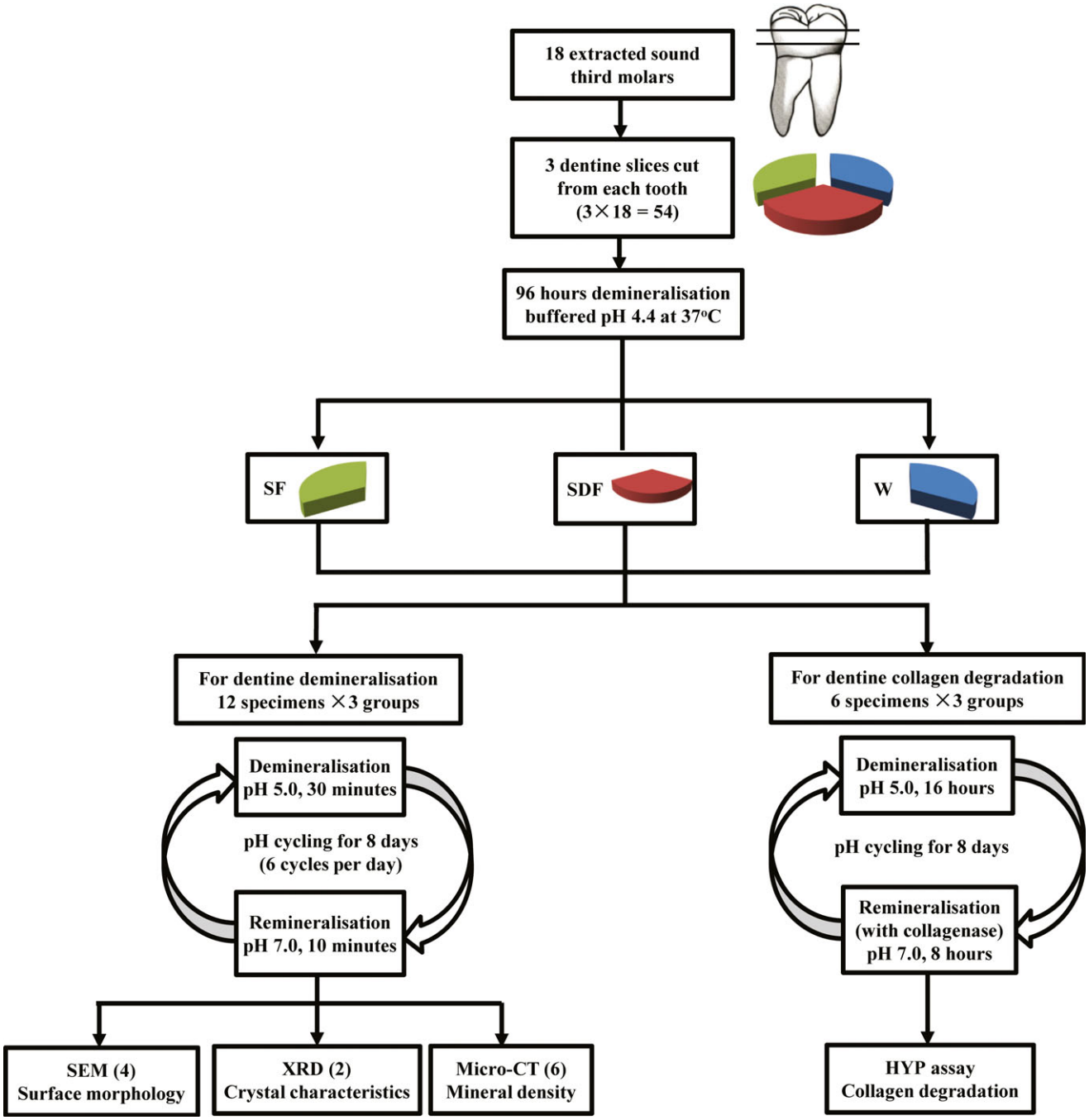

Figure 1. Flow chart of the experimental design.

They were sectioned to make dentine slices of 2 mm thickness. A total of 18 dentine slices were prepared from 18 human third molars. Microfine 4,000-grit sandpaper was used to polish the surfaces of the dentine slices. A stereomicroscope was used to examine the polished dentine slices to ensure that they had no cracks or other defects. Each dentine slice was then sectioned into three specimens. A total of 54 specimens were prepared. An acid-resistant nail polish (Clarins, Paris, France) was used to cover half of the treatment surface of the specimens. The specimens were then sterilised with ethylene oxide before they were put into a demineralisation solution [50 mM acetate, 2.2 mM calcium chloride ($CaCl_2$), 2.2 mM monopotassium phosphate (KH_2PO_4), pH 4.4] for 96 hours at room temperature. This protocol created demineralised lesions of 70–100 μm depth on the specimens[7]. The specimens were washed with deionised water after development of the demineralised lesion. Subsequently, 36 specimens (from 12 dentine slices) were used for the dentine demineralisation test and 18 specimens (from six dentine slices) were used for the dentine collagen-degradation test.

Experimental treatment

The three specimens from each dentine slice were distributed among the three treatment groups. In the first group (group SF), the specimens received topical application of a single drop of 25% $AgNO_3$ solution containing 151,130 p.p.m. silver (SILVER NITRATE liquid; Gordon Laboratories, Upper Darby, PA, USA), followed by 5% NaF varnish containing 22,600 p.p.m. fluoride (Duraphat in a 10 ml tube; Colgate-Palmolive Co., New York City, NY, USA). In the second group (group SDF, the positive control), the specimens underwent a single topical application of a 38% SDF solution (Cariostop, Biodinamica, Brazil) containing 253,900 p.p.m. silver and 44,800 p.p.m. fluoride. In the third group (group W, the negative control), the specimens were treated with deionised water. The bottles of the experimental solutions were shaken before use. A microbrush (Micro applicator – regular; Premium Plus International Ltd., Hong Kong, China) was used to apply all solutions to the surface of the specimen. In our pilot experiment, about 17±2 μl of SDF or NaF was delivered in a single application to the specimen. All the treated specimens were kept at room temperature for 30 minutes before pH cycling.

Assessment of dentine demineralisation

pH cycling for dentine demineralisation

The pH cycling was employed on specimens by demineralisation for 30 minutes and remineralisation for 10 minutes (*Figure 1*). The experiment duration was six cycles per day for 8 days. The compositions of demineralisation and remineralisation solutions were as follows: demineralisation solution: 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 50 mM acetate, pH 5.0; and remineralisation solution: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4 and 150 mM potassium chloride (KCl), pH 7.0. Specimens were stored at 4 °C in deionised water during the non-treatment period[7].

Surface morphology

Four specimens from each group were fixed in 2.5% glutaraldehyde at 4 °C for 4 hours. They were washed in distilled water and then dehydrated in a series of ethanol solutions (70% for 10 minutes, 95% for 10 minutes and 100% for 20 minutes). The specimens were then critical-point dried in a desiccator and sputter-coated with carbon. Scanning electron microscopy (Hitachi S-4800 FEG Scanning Electron Microscope; Hitachi Ltd., Tokyo, Japan) was used to examine the surface morphology of the specimens.

Crystal characteristics

X-ray diffraction (XRD) data were collected from each group using an X-ray diffractometer (Bruker D8 Advance; Bruker AXS, Karlsruhe, Germany), with CuKa (l = 1.5418 Å) radiation, equipped with a scintillation counter. These data were collected with a range of 20–60° 2q, a step size of 0.05° and a scan speed of 30 seconds/step. The diffraction data were re-collected to reduce systematic errors after a preliminary data collection. International Centre for Diffraction Data (ICDD, PDF-2 Release 2004) was used as the database to check the phase purity and indexing of the chemical phase. The Bruker DIFFRAC plus EVA program was used to analyse the diffraction patterns[8].

Lesion depth

Micro-computed tomography (micro-CT) (SkyScan 1076; SkyScan, Antwerp, Belgium) was used to scan the specimens for measurement of lesion depth. The signal-to-noise ratio was 5. The highest spatial scanning resolution was 9 μm. A 1-mm aluminum filter was used to remove the softest X-rays. The voltage and current of the X-ray source were 100 kV and 80 μA, respectively. The scanning results were reconstructed using software NRecon reconstruction (SkyScan). Data-analysing software CTAn (SkyScan) was used to view and process the reconstructed three-

dimensional images. From the reconstructed image of each specimen, cross-sectional images were located. From these lesion images, 10 were randomly selected. The lesion depth of different groups was measured using image analysis software (Image J; National Institutes of Health, Bethesda, MD, USA), with internal control as a reference line[7]. Six specimens per group were assessed.

Assessment of dentine collagen degradation

The presence of the non-proteinogenic amino acid hydroxyproline (HYP) was measured to estimate the degradation of dentine collagen[8]. The pH cycling was employed on specimens with the above-mentioned demineralisation solution for 16 hours and a remineralisation solution [1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 20 mM HEPES, 150 mM KCl, 1.5 U/ml of highly purified collagenase type VII from *Clostridium histolyticum* (C-0773; Sigma Chemical Co., St Louis, MO, USA)] at pH 7.0 for 8 hours at 37 °C, for 8 days (*Figure 1*)[4]. The pooled remineralisation solutions from pH cycling were concentrated using a Savant SpeedVac Concentrator (Thermo Scientific, Waltham, UK), and rehydrolysed at 120 °C for 20 minutes. Subsequently, a buffered 0.056 M chloramine-T reagent was added to the hydrolysed samples. The samples were kept at room temperature for 25 minutes to allow oxidation. Then, 1 M Ehrlich's reagent was added for development of the chromophore. The mixture was then incubated at 65 °C for 20 minutes. The absorbance was read at 550 nm (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA) spectrophotometrically. A standard solution containing 2–20 μg of HYP was also tested to generate the standard curve[7]. The standard curve had a coefficient of determination of 0.93. Six specimens per group were assessed.

Statistical analysis

The Shapiro–Wilk test of normality ($P > 0.05$) was used to assess whether the data had a normal distribution. One-way analysis of variance with Bonferroni multiple comparison tests was used to compare the lesion depth and concentration of HYP in the remineralisation solutions across the three treatment groups. Analyses were performed with the computer software SPSS Statistics – V20.0 (IBM Corporation, Armonk, NY, USA). The level of statistical significance for all tests was set at 0.05.

RESULTS

Scanning electron microscopy revealed that collagen fibres were not exposed on dentine surfaces, and that the surfaces were relatively smooth, in groups SF and SDF (*Figure 2*a,c). There was limited space remaining in inter-tubular and intra-tubular areas (*Figure 2*b,d). Cross-sectional images showed an irregular squamous layer of material deposits, with a depth of approximately 1 μm, on the surface of the specimens in group SF. This thin layer partially plugged many dentinal tubules (*Figure 3*a,b). Dense granular structures of spherical grains were observed in the inter-tubular area in both group SF and group SDF (*Figure 3*b,d). However, this structure was absent in group W. Exposed collagen was sparsely and distinctly distributed in group W (*Figure 2*e,f). This observation was also confirmed by the cross-sectional images (*Figure 3*e,f).

Typical XRD spectra of the three groups are shown in *Figure 4*. The results show that the crystal composition on the dentine surfaces corresponded to hydroxyapatite (HAP) in all groups. In group W, the diffraction peaks at 31.8° (211) and 33.3° (300) broadened; this 'amorphous' pattern indicates the loss of crystallinity of dentine as a result of dissolution of the HAP crystal structure. Apart from HAP, the strong peaks at 27.8°, 32.2°, 46.2°, 54.8° and 57.5° in groups SF and SDF were coincident with silver chloride (AgCl) (111), (200) and (220) Bragg reflections, which suggests that AgCl was formed. In addition, there was clearly an additional peak of Ag (111) in groups SF and SDF, which suggests the formation of metallic silver[9].

Images of typical micro-CT in treatment groups are shown in *Figure 5*. The lesion depths in group SF and group SDF were less than that in group W. This was confirmed by measuring the depth of the carious lesions (*Figure 6*). The mean lesion depth (±SD) was 128 ± 19 μm in group SF and 135 ± 24 μm in group SDF; these were significantly less than the lesion depth for group W (258±53 μm) ($P < 0.05$). The HYP concentration (±SD) in the remineralisation solution was significantly higher in group W (339 ± 16 μg/ml) than in groups SF (312 ± 11 μg/ml) and SDF (317±16 μg/ml) (*Figure 7*).

DISCUSSION

This study used 38% SDF as a positive control. Clinical studies have shown that 38% SDF prevents and arrests coronal caries in preschool children[10,11] and root caries in elderly subjects[12,13]. Laboratory studies have found that SDF has a strong antibacterial effect on cariogenic biofilm[8,14] and a potent inhibitory effect on the activity of matrix metalloproteinases[4] and cysteine cathepsins[15]. Treatment with SDF can increase the mineral density of enamel carious lesions[16] and the microhardness of dentine carious lesions[17]. An *in vitro* study found that the principal components of

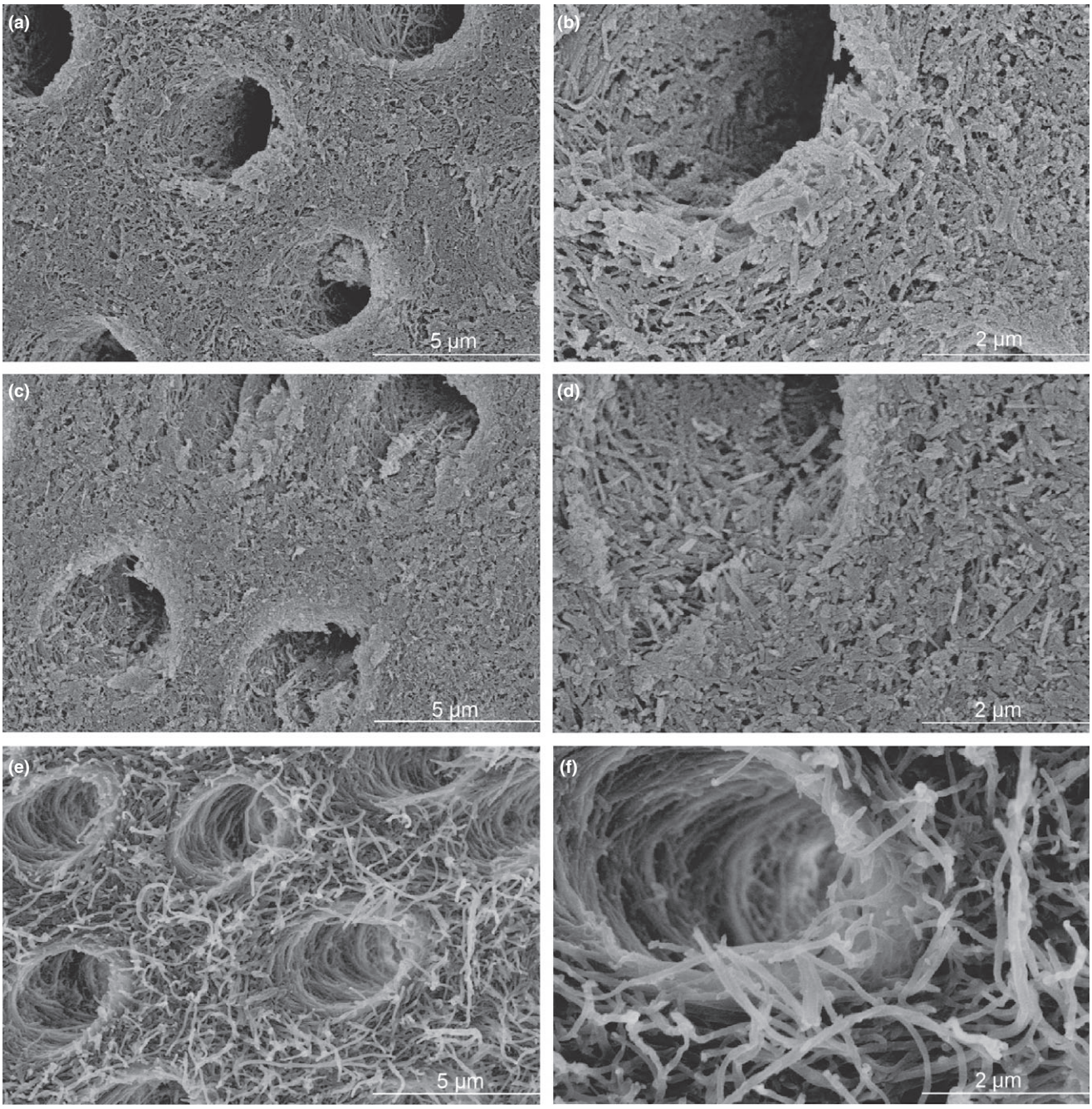

Figure 2. Scanning electron microscopy images of the surface morphology of artificial dentine caries. (a) 8,000× and (b) 20,000× magnification views of the group treated with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish (group SF); (c) 8,000× and (d) 20,000× magnification views of the group treated with topical application of 38% silver diamine fluoride solution (group SDF); (e) 8,000× and (f) 20,000× magnification views of the group treated with deionized water (group W).

tooth tissue react with SDF to form calcium fluoride, which has a caries-protective effect[18]. Another laboratory study reported that SDF could inhibit demineralisation and preserve dentine collagen from degradation in demineralised dentine[7]. However, SDF is not available in certain countries, including the UK. A promising caries-arrest rate of human carious lesions was recently reported following application of $AgNO_3$ followed by NaF[1]. This protocol can easily be achieved by using two commercial products, namely 25% $AgNO_3$ solution and 5% NaF varnish, which are readily available worldwide. The aim of the present study was to investigate the chemical and histological changes in simulated dentine carious lesions in order to understand the mechanism of action on caries arrest.

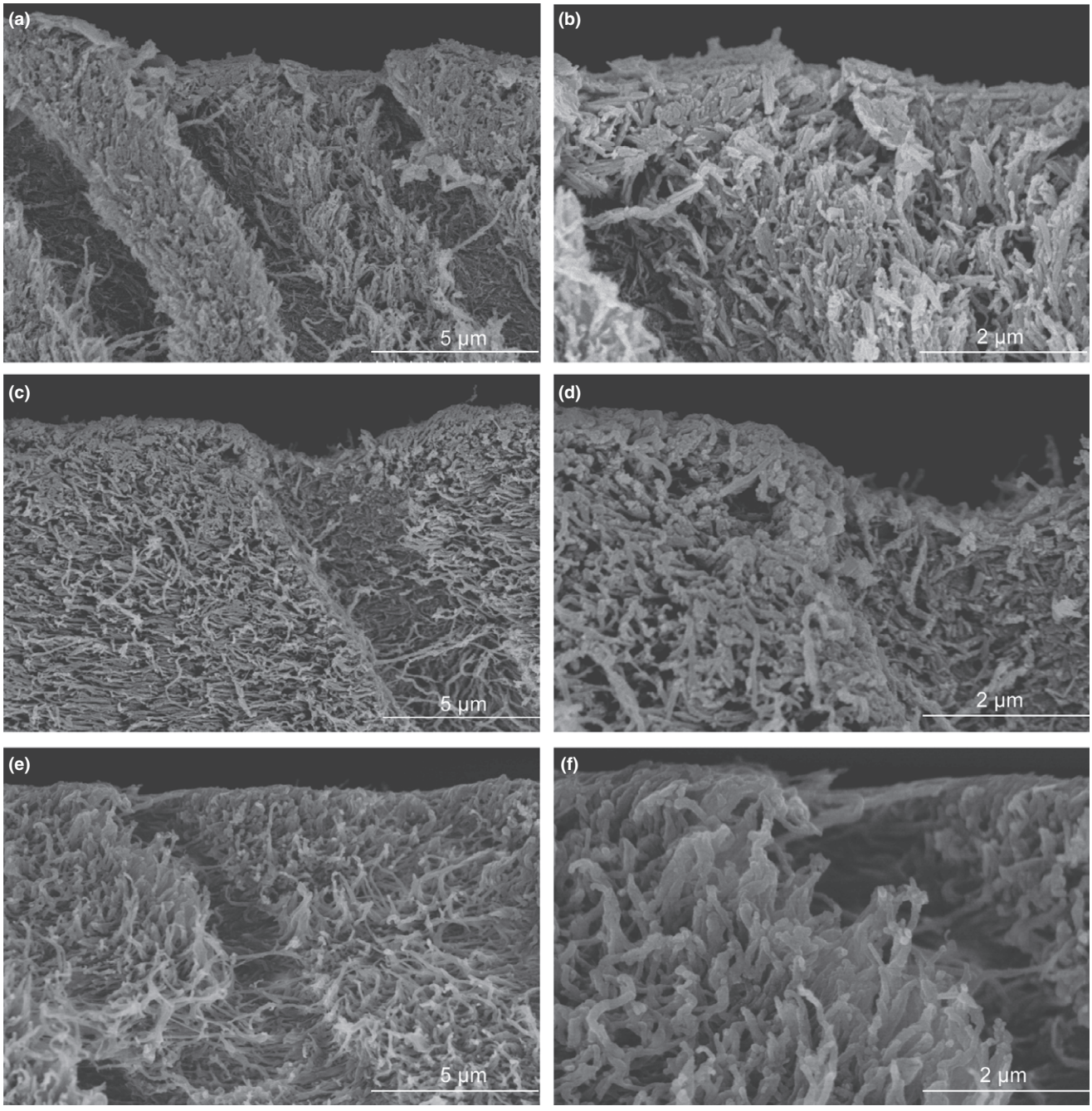

Figure 3. Scanning electron microscopy images of the cross-section of artificial dentine caries. (a) 8,000× and (b) 20,000× magnification views of the group treated with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish (group SF); (c) 8,000× and (d) 20,000× magnification views of the group treated with topical application of 38% silver diamine fluoride solution (group SDF); (e) 8,000× and (f) 20,000× magnification views of the group treated with deionized water (group W).

The model of the present study adopted the same pH-cycling model used in our previous publication[7]. This model simulated the pH associated with the natural dental-caries process and dynamic variation in mineral saturation. Briefly, substantial demineralisation and the development of a thick layer of demineralised organic matrix are expected to assess dentine collagen degradation. Thus, a long duration of incubation with bacterial collagenase was used in the dentine collagen-degradation experiment, to ensure that an adequate amount of HYP was liberated as a result of the degradation of dentine collagen[5,7]. This study is a laboratory study based on a chemical model and is very different from complex clinical conditions. The results cannot be extrapolated to the *in vivo* situation and caution should be exercised in their interpretation.

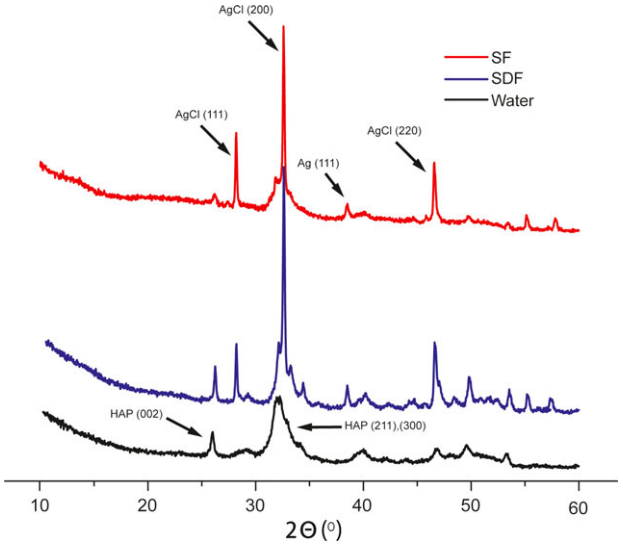

Figure 4. Typical X-ray diffraction (XRD) patterns of the experimental groups. SDF, treatment with topical application of 38% silver diamine fluoride solution; SF, treatment with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish; W, treatment with deionized water.

Dense granular structures of spherical grains in the inter-tubular area were formed on the surfaces of the specimens treated with $AgNO_3$ solution and NaF varnish (i.e. the SF group), as well as on the surfaces of the specimens treated with SDF. This indicates the formation of extrafibrillar mineral in these two groups. From the cross-sectional images, an irregular squamous layer was observed on the surface of the specimens treated with $AgNO_3$ solution and NaF varnish, which is different from the specimens treated by SDF. This formation of a squamous layer might be a result of the natural resin of the fluoride varnish. According to the information provided by the company, the composition of the varnish is NaF in an alcoholic solution of natural resins. Natural resin is soluble in alcohol but not in water. Therefore, it can protect the surface from water intrusion. Duffin[1] suggested that applying fluoride varnish over the area treated with $AgNO_3$ would have multiple benefits, such as preventing contact of the $AgNO_3$ with the soft tissue and providing a protective layer to keep the $AgNO_3$ from being washed away by saliva. The specific chemical reactions are suggested below:

$$2Ca_5(PO_4)_3OH + 20Ag^+ \rightarrow 6Ag_3PO_4 + 10Ca^{2+} + Ag_2O + H_2O \quad (1)$$

$$Ca_5(PO_4)_3OH + F^- \rightarrow Ca_5(PO_4)_3F + OH^- \quad (2)$$

$$Ca_5(PO_4)_3OH + 20F^- \rightarrow 10CaF_2 + 6PO_4^{3-} + 2OH^- \quad (3)$$

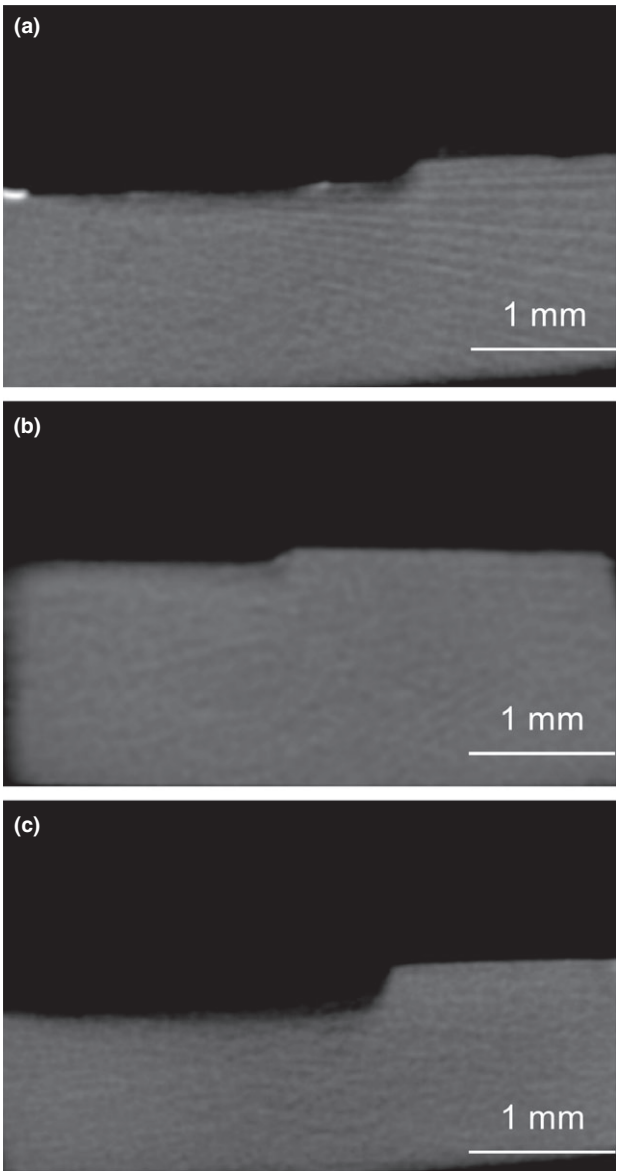

Figure 5. Typical micro-computed tomography images of the cross-section of artificial dentine caries, left side: lesion body; right side: internal control. (a) Treatment with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish (group SF); (b) treatment with topical application of 38% silver diamine fluoride solution (group SDF); (c) treatment with deionized water (group W).

$$Ca^{2+} + F^- \rightarrow CaF_2 \quad (4)$$

In (1), the solubilities of silver phosphate (Ag_3PO_4) (6.5×10^{-4} g/100 ml) and silver oxide (Ag_2O) (1.3×10^{-3} g/100 ml) are higher than that of AgCl (8.9×10^{-5} g/100 ml). Thus, Ag_3PO_4 and Ag_2O could react with solutions of alkali chlorides to form AgCl. This could explain why AgCl was the major precipitate detected by XRD in this study. In (2), both $Ca_5(PO_4)_3OH$ (HAP) and $Ca_5(PO_4)_3F$ (fluorapatite, FAP) are the main components of human teeth[19].

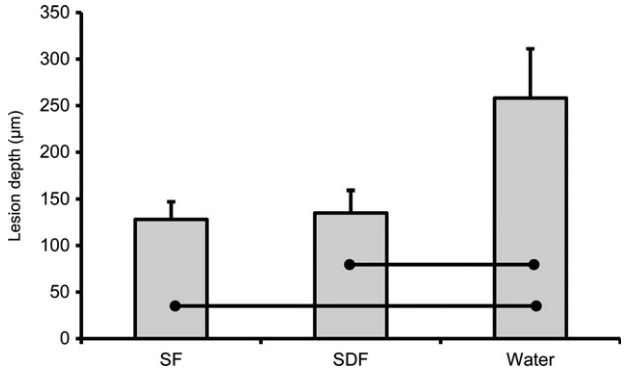

Figure 6. Lesion depths of the three experimental groups. Columns linking bars with markers indicate significant differences at $P < 0.05$ between groups. SDF, treatment with topical application of 38% silver diamine fluoride solution; SF, treatment with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish; W, treatment with deionized water.

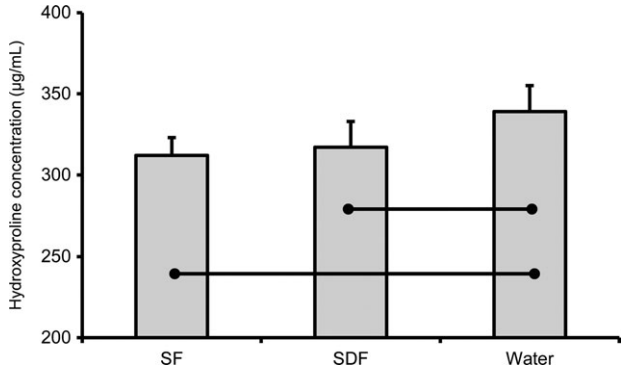

Figure 7. Hydroxyproline concentrations of the three experimental groups. Columns linking bars with markers indicate significant differences at $P < 0.05$ between groups. SDF, treatment with topical application of 38% silver diamine fluoride solution; SF, treatment with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish; W, treatment with deionized water.

When HAP is exposed to a low concentration of fluoride in an acidic environment, FAP is formed because the solubility of FAP is lower than that of HAP[20]. Given that the pH of the NaF varnish is 4.5[21], this reaction has a high chance of proceeding. In (3) and (4), calcium fluoride (CaF_2) is an important product that is produced when fluoride is deposited onto the tooth surface. CaF_2 can act as a temporary fluoride reservoir and can release fluoride ions at low pH[22]. The F^- released might facilitate formation of FAP. In this study, we could not detect CaF_2 using XRD and therefore we considered that a negligible amount of CaF_2 was present, possibly removed by washing with water[18]. Moreover, the signal of CaF_2 would have been weakened when it coexisted with heavy atoms, such as silver. HAP and FAP are both calcium apatites, and the size of OH^- is similar to that of F^-. Their chemical structures might be highly similar, explaining the very slight difference found in XRD spectra[23]. Dentine is a biomaterial comprising both mineral and organic components, and the latter could also affect the spectrum. This difference between HAP and FAP is below the detection threshold of XRD[24]. Nevertheless, we found the formation of typical clustered granular structures of spherical grains under scanning electron microscopy and smaller lesion depth than the control group, suggesting that remineralisation of the demineralised dentine had occurred after adjunctive application of $AgNO_3$ solution and NaF varnish. This could explain the clinical success in caries arrest.

HYP is a major component of collagen I and plays crucial roles in collagen stability[5]. When dentine degrades, insoluble collagen molecules become soluble collagen, and HAP is released. The estimation of HYP concentration was used for direct evaluation of degraded collagen in solution[25]. Our previous study found that SDF could protect dentine collagen against bacterial proteolysis[7]. Likewise, this study found that using $AgNO_3$ solution and NaF varnish could inhibit degradation of dentine collagen in terms of HYP content. Silver ions have been suggested to inactivate their catalytic functions by interacting with a reactive side chain of the bacterial collagenase[26]. The large ionic radius and low oxidation state of silver ions have been shown to have strong affinity to protein[27] and this may contribute to the inhibitory effect of silver ions on bacterial collagenase. The metal ion probably interacts with a reactive side chain of the enzymes to inactivate their catalytic functions. It is plausible that the silver ions in the $AgNO_3$ solution interacted with exposed collagen in demineralised dentine and thus inhibited the activity of bacterial collagenase. This mechanism might have contributed to the results of lesion depth and HAP content.

It is noteworthy that the concentrations of silver and fluoride in 25% $AgNO_3$ and 5% NaF, respectively, are 151,130 p.p.m. and 22,600 p.p.m., and approximately half of the concentration of silver and fluoride in 38% SDF (253,900 p.p.m. silver and 44,800 p.p.m. fluoride, respectively)[28]. No significant difference in this *in vitro* study was found for dentine demineralisation and dentine collagen degradation between use of 38% SDF and adjunctive application of 25% $AgNO_3$ and 5% NaF. We are performing a clinical trial (ClinicalTrials.gov Identifier: NCT02019160) to determine whether 25% $AgNO_3$ and 5% NaF are as effective as 38% SDF in arresting childhood caries. The concentrations of silver and fluoride are lower in 25% $AgNO_3$ and 5% NaF, respectively, than in 38% SDF; therefore, 25% $AgNO_3$ and 5% NaF could be more favourable for use in young children when considering the side effects of high concentrations of silver and fluoride[29]. Because its use for caries management is painless, simple, low-cost and approved in many countries[30,31],

25% $AgNO_3$ with 5% NaF could be widely recommended and promoted as an alternative treatment of invasive caries to that of conventional management of caries, particularly among child patients who are too young for conventional dental care.

CONCLUSION

The results of the *in vitro* study indicate that the use of $AgNO_3$ solution and NaF varnish is effective in inhibiting dentine demineralisation and dentine collagen degradation. The application of $AgNO_3$ solution and NaF varnish could be an alternative to SDF in treating dentine caries.

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Conflict of interest

The authors declare that they have no conflict of interests.

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