Inhibitory effect of silver diamine fluoride on dentine demineralisation and collagen degradation

Short title: Silver diamine fluoride on dentine remineralisation

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Abstract

Objective: To investigate the inhibitory effects of 38% silver diamine fluoride (SDF) on demineralised dentine.

Methods: Human dentine blocks were demineralised and allocated to four groups: SF, F, S and W. The blocks in group SF received a topical application of 38% SDF solution (253,900 ppm Ag, 44,800 ppm F), group F received a 10% sodium fluoride solution (44,800 ppm F), group S received a 42% silver nitrate solution (253,900 ppm Ag) and group W received deionised water (control). They were subjected to pH cycling using demineralisation solution (pH 5) and remineralisation solution (pH 7) for 8 days. The surface morphology, crystal characteristics, lesion depth and collagen matrix degradation of the specimens were investigated by scanning electron microscopy (SEM), x-ray diffraction (XRD), micro-CT testing and spectrophotometry with a hydroxyproline assay.

Results: The surface morphology under SEM showed evident demineralisation with exposed collagen in groups S and W, but not in group SF. Clusters of granular spherical grains were observed in the cross-sections of specimens in groups SF and F. XRD revealed precipitates of silver chloride in groups SF and S. The mean lesion depth ($\pm$SD) of groups SF, F, S and W were 182 ± 32 μm, 204 ± 26 μm, 259 ± 42 μm and 265 ± 40 μm, respectively (SDF, F<S, W; $p<0.01$). Groups SF and S had significantly less hydroxyproline liberated from the dentine matrix than groups F and W ($p<0.01$).

Conclusion: The use of 38% SDF inhibited demineralisation and preserved collagen from degradation in demineralised dentine.

Clinical significance: SDF application positively influences dentine remineralization.

1.   Introduction

A variety of chemical agents has been used in clinical trials or *in vitro* studies to arrest dentine caries. Some antimicrobial agents contain silver (Ag), which is bactericidal.[1] The first widely reported silver-containing agent for arresting dentine caries was silver nitrate ($AgNO_3$), and 25% silver nitrate is used to arrest carious lesions in the US.[2] Topical fluoride (F) agents such as sodium fluoride (NaF) and silver diamine fluoride (SDF) are also used for arresting caries.[3] A literature review for SDF concluded that it is effective in preventing and arresting coronal and root caries.[4] Another review concluded that SDF is a safe, effective, efficient and equitable caries-preventive agent that appears to meet the criteria of the WHO Millennium Goals and the US Institute.[5] While studies report clinical success with SDF in arresting dental caries,[5] laboratory studies have found that SDF has an intense antibacterial effect on cariogenic biofilm, and hinders caries progression.[6-9]

While Liu and her co-workers[10,11] demonstrated that SDF increased the mineral density of demineralised enamel lesions in a mineralising solution, the inhibitory effect of SDF on demineralised dentine is yet to be studied. Acid attacks generate demineralised dentine. The acid can arise from bacteria (causing dental caries), food and beverages (causing dental erosion) or even from acid etching during dental procedures. Regarding dental caries, enamel caries are basically the dissolution of minerals by bacterial acids, whereas dentine caries are a complex progression involving both demineralisation and organic degradation.[12] The organic matrix is exposed once a dentine surface is demineralised. Fibrillar type I collagen accounts for 90% of the organic matrix while the remaining 10% consists of non-collagenous proteins. The remineralisation process should be controlled through the interactions of mineral crystallites with the collagen matrix. The remineralisation process requires not only a sufficient supply of calcium and phosphate ions but also the preservation of the integrity of the dentine collagen, which acts as a scaffold for mineral deposition.[13,14] Therefore, this study aimed to investigate the mechanism of SDF in the remineralisation of demineralised dentine from both mineral and collagen aspects. The null hypothesis was that there is no difference in the inhibitory effect on dentine demineralisation and collagen degradation of 38% SDF solution (253,900 ppm Ag and 44,800 ppm F), 10% NaF solution (44,800 ppm F), 42% $AgNO_3$ solution (253,900 ppm Ag) and deionised water.

2.   Materials and methods

2.1   Preparing specimens with artificial dentine caries

This study was approved by the Institutional Review Board (IRB UW08-052). The flow chart in Figure 1 summarises the protocol of this study. Extracted sound human molars were collected with patient consent. Teeth were stored in distilled water at 4°C until use and were used within one month of extraction. Eighteen dentine blocks with thicknesses of 2 mm were prepared from 18 molars. The surfaces of the blocks were polished using micro-fine 4,000 grid sanding paper. The polished slices were examined using a stereomicroscope to exclude samples with cracks or other defects. Each slice was cut into four specimens for different treatments afterwards. Therefore, 72 specimens were prepared.

All of the specimens were half-varnished with an acid-resistant nail polish (Clarins, Paris, France). They were immersed in a demineralisation solution (2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, 50 mM acetate) at pH 4.4 for 96 h at 25°C to create lesions 70-100 μm deep (confirmed by pilot study, data not shown). Following lesion development, the specimens were rinsed thoroughly with deionised water. [15]

2.2   Experimental treatment

The four specimens from each dentine slice were allocated to the four treatment groups. In group SF, the specimens underwent a topical application of a 38% SDF solution (Saforide; Toyo Seiyaku Kasei Co. Ltd., Osaka, Japan). According to the manufacturer's instructions, the solution contained 253,900 ppm Ag and 44,800 ppm F. In group F, the specimens were treated with a freshly prepared 10% NaF solution containing 44,800 ppm F. In group S, the specimens were treated with a freshly prepared 42% $AgNO_3$ solution containing 253,900 ppm Ag. Group W was the control group in which the specimens were treated with deionised water. All solutions were applied to the specimen surface with a micro-brush (Micro applicator – regular, Premium Plus International Ltd., Hong Kong, China) and the average amount of solution applied was 0.22 mg.[7]

2.3   Assessment of dentine demineralisation

2.3.1   pH cycling for dentine demineralisation

To assess the effect of the solutions on dentine demineralisation in the four treatment

groups, the protocol used by Xie and his co-worker[15] was employed. In brief, 12 specimens of each group were pH cycled at room temperature through a 30 min immersion in demineralisation solution (1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 50 mM acetate) at pH 5.0 followed by a 10 min immersion in remineralisation solution (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 150 mM KCl) at pH 7.0. Six cycles per day were performed for 8 days. All of the solutions were freshly prepared for use in the experiment. Specimens were kept in deionised water at 4°C overnight.[15]

2.3.2 Surface morphology

Four specimens in each group were fixed in 4% (vol/vol) formaldehyde. They were then washed in distilled water ultrasonically three times, dehydrated in a series of ethanol solutions, critical-point dried in a desiccator and finally sputter-coated with carbon. The surface morphologies of the specimens were then examined under scanning electron microscopy (SEM) (Hitachi S-4800 FEG Scanning Electron Microscope, Hitachi Ltd., Tokyo, Japan) at 5 kV in high-vacuum mode.[7]

2.3.3 Crystal characteristics

Step-scanned lock-coupled x-ray diffraction (XRD) data were collected from two specimens from each group by a Bruker D8 Advance x-ray powder diffractometer with CuKa (l = 1.5418 Å) radiation equipped with a scintillation counter. The accelerating voltage and the applied current of the x-ray generator were 40 kV and 40 mA, respectively. The x-ray beams were paralleled via a Göbel Mirror and confined by a divergence-limiting slit (0.6 mm) and a Soller slit before reaching the sample to reduce the axial divergence of the incident beam. A receiving slit (0.6 mm) and a detector slit (0.2 mm) were employed to increase resolution. The data collection parameters were as follows: 2q range = 20–60°, step size = 0.05° and scan speed = 30 second/step. After a preliminary data collection, the diffraction data were re-collected to minimise systematic errors. A reproducible dataset for each sample was obtained and the sample damage by x-ray irradiation was negligible. The phase purity and indexing of the chemical phase were checked by a database match search using the International Centre for Diffraction Data (ICDD, PDF-2 Release 2004). The diffraction patterns were analysed using the Bruker DIFFRACplus EVA program.[16]

2.3.4 Lesion depth

The specimens (n=6 for each group) were scanned by a SkyScan 1076 micro-CT

(SkyScan, Antwerp, Belgium) for lesion depth assessment. The x-ray source was operated at a voltage of 100 kV and a current of 80 kA. The highest spatial resolution used for the scanning was 9l m. A signal-to-noise ratio of 5 was chosen, and a 1 mm aluminium filter used to cut off the softest x-rays. The scanning results for each specimen were reconstructed using the NRecon reconstruction software (SkyScan, Antwerp, Belgium). The reconstructed 3-D images were viewed and processed using the data analysing software CTAn (SkyScan, Antwerp, Belgium). From the reconstructed 3-D image of each specimen, cross-sectional images in each specimen were located.[11] From these lesion images, 10 were randomly selected. The lesion depth was measured using special image analysis software (Image J; National Institutes of Health, USA).

2.4   *Assessment of dentine collagen degradation*

To assess the effect of the solutions on collagen degradation in the four treatment groups, the protocol used by Hiraishi and her co-worker[13] was employed. In brief, 6 specimens of each group were pH cycled through the abovementioned demineralisation solution for 16 h and a remineralisation solution (20 mM HEPES, 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 150 mM KCl, 1.5 U/mL highly purified collagenase type VII from *Clostridium histolyticum* (C-0773, Sigma Chemical Co., St. Louis, MO, USA)) at pH 7.0 for 8 h at 37°C for 8 days. All of the solutions were freshly made prior to use.[13]

Collagen degradation was determined by estimating the presence of the non-proteinogenic amino acid hydroxyproline (HYP).[13,17,18] Measurement of HYP in each sample was determined in triplicate by quantitative analysis, as described elsewhere,[19] with minor modifications. Briefly, the pools of remineralisation solution collected from pH cycling were concentrated at a high drying rate using a Savant SpeedVac Concentrator (Thermo Scientific, Waltham, UK), and re-hydrolysed by autoclaving at 120°C for 20 min. The hydrolysed samples were then mixed with a buffered 0.056 M chloramine-T reagent, and oxidation was allowed to proceed for 25 min at room temperature. The chromophore was then developed with the addition of 1M Ehrlich's reagent and incubated at 65°C for 20 min. A HYP standard solution containing 2-20 µg HYP was also prepared. The absorbance was spectrophotometrically read at 550 nm (SpectraMax 340, Sunnyvale, CA, USA) and corrected for the reagent blank. The standard curve had a coefficient of determination of 0.99.

2.5   *Statistical analysis*

All of the data were assessed for a normal distribution using the Shapiro-Wilk test for

normality ($p>0.05$). A one-way ANOVA was used to compare the lesion depth and concentration of HYP in the remineralisation solutions across the four treatment groups. All of the analyses were conducted using IBM SPSS Version 2.0 software (IBM Corporation, Armonk, New York, USA). The cut-off level for significance was taken as 5% for all of the analyses.

3. Results

We observed that dentine collagen fibres were not exposed on the relatively smooth surface of the specimens in group SF, (Figure 2a) and only a little space remained in both inter-tubular and intra-tubular areas (Figure 2b). Cross-sectional images showed the dense granular structures of spherical grains in the inter-tubular area (Figures 3a and 3b). Group F showed a relatively rough surface (Figure 2c) with some exposed collagen and an increased inter-fibrillar distance (Figure 2d). Cross-section images confirmed these findings (Figures 3c and 3d). In groups S and W, there was evidence of the exposure of the reticular nanostructure of the collagen fibres on the surface, showing the effect of partial demineralisation (Figures 2e and 2g). Exposed fibres were evident in both inter-tubular and intra-tubular areas under higher magnification (Figures 2f and 2h). Likewise, exposed collagen was distinct and sparsely distributed in cross-sectional images (Figures 3e and 3g). There was little granular structure with spherical grains (Figures 3f and 3h).

Typical XRD spectra of the four groups are shown in Figure 4. The XRD analysis indicates that the crystal composition on the dentine surfaces in all of the groups corresponded to hydroxyapatite (HAP), which is crystallised in the (002), (211) and (300) Bragg reflections. Apart from HAP, the strong peaks at 27.8°, 32.2° and 46.2° in groups SF and S were coincident with silver chloride (AgCl) (111), (200) and (220), which suggests that AgCl was formed on the surfaces in these two groups. In addition, there was clearly a cubic phase of Ag (111) in group SF, which suggests the formation of metallic silver.[20]

The depth of the carious lesions in the treatment groups are shown in Figure 5. The mean lesion depth ($\pm$SD) of groups SF and F were 182$\pm$32 µm and 204$\pm$26 µm, respectively, significantly less than those of groups S and W, which were 259$\pm$42 µm and 265$\pm$40 µm, respectively. The concentration of HYP ($\pm$SD) in the remineralisation solution, which is directly proportional to the amount of degraded collagen, was significantly higher in groups SF (346$\pm$57 µg/mL) and S (349$\pm$18 µg/mL) than in groups F (189$\pm$85 µg/mL) and W (469$\pm$63

μg/mL) (Figure 6).

4. Discussion

This study investigated the effect of SDF on the inorganic (mineral) and organic (collagen) content of demineralised dentine. It provides useful information about the microstructural changes in the minerals and collagen of demineralised dentine. According to the results of this study, the null hypothesis was rejected. SDF showed a higher inhibitory effect on both dentine demineralisation and collagen degradation. This could be the reason for its success in arresting caries in clinical trials.

The dentine specimens were initially demineralised to simulate dentine carious lesions. Then pH cycling models were designed to mimic the dynamic variations in mineral saturation and pH associated with the natural caries process.[13,15] We used two different pH cycling models to assess the effects of SDF on demineralisation and collagen degradation, respectively. To assess the mineral changes, pH cycling with six cycles per day was adopted. This model is commonly used in caries remineralisation studies.[15,21,22] However, to assess collagen degradation, extensive demineralisation and the development of a relatively thick layer of demineralised organic matrix were anticipated. A longer incubation period with bacterial collagenase was chosen so that the amount of degradation would be adequate for HYP assessment.[13,18] The dentine specimens were fixed in formaldehyde to stable the collagen before SEM observation, formaldehyde may produce formic acid which can demineralise the dentine specimens, thus dissolving minerals on the surface. Karnovsky glutaraldehyde could be an alternative fixative.

To remineralise demineralised dentine, three conditions are necessary.[14] Firstly, there should be residual mineral crystals to serve as growth centres. Secondly, there should be a supply of mineral sources containing calcium and phosphorus. Finally, the collagen structure should be sound to serve as a scaffold for the mineral crystals to grow on. An environment that favours the precipitation of calcium and phosphorus is therefore essential for the remineralisation of demineralised dentine. Furthermore, intact dentine collagen should be present. Studies have attempted to remineralise initial coronal caries and root caries through the application of calcium phosphate, bioglass and fluoride to enhance mineral deposition.[23-25] There are also studies using matrix metalloproteinase (MMP) inhibitors such as chlorhexidine

to protect the collagen from degradation.[14,26] Hesperidin, which interacts with collagen and/or non-collagenous proteins, has also been used to stabilise the collagen matrix and induce remineralisation.[13]

A dense granular structure of spherical grains was observed under SEM on the surface of the demineralised dentine treated with SDF, which indicates extra-fibrillar mineral formation.[27] Alternatively, transmission electron microscopy can be performed to investigate the mineral. Our earlier study found that the micro-hardness of remineralised dentine increased after SDF treatment.[6] It is plausible that the remineralisation of the demineralised dentine occurred in both an extra-fibrillar and intra-fibrillar manner. It is noteworthy that demineralised dentine specimens are partially demineralised after immersion in a demineralising solution; the HAP residues could act as sites for apatite nucleation and the growth of HAP in the presence of calcium and phosphate, [27]enabling remineralisation and recovery of the mechanical properties of the demineralised dentine.

It is suggested that silver phosphate (Ag_3PO_4) and silver oxide (AgO_2) are formed when $AgNO_3$ reacts with the HAP of major tooth components ($Ca_{10}(PO_4)_6(OH)_2$.[1] The succinct chemical reaction below is suggested.

$$Ca_{10}(PO_4)_6(OH)_2 + 20\ AgNO_3 \longrightarrow 6\ Ag_3PO_4 + 10\ Ca(NO_3)_2 + Ag_2O + H_2O$$
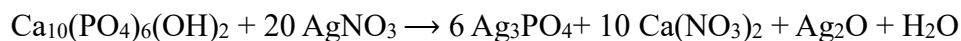
Ag_2O is insoluble but can be dissolved in an ammonia solution to give soluble derivatives. It also reacts with solutions of alkali chlorides to precipitate silver chloride (AgCl). Ag_3PO_4 also reacts with solutions of alkali chlorides to form AgCl. The solubility product of AgCl (6.5×10^{-4} g/100 ml) is lower than that of Ag_3PO_4 (8.9×10^{-5} g/100 ml). This may be the reason that AgCl was the principal precipitate detected by XRD in this study. $Ca(NO_3)_2$ is soluble and can be dissolved in saliva once formed. This loss of Ca from the dentine intensifies the loss of minerals and exposes the collagen, increasing the demineralisation after $AgNO_3$ application. In addition, the increase in demineralisation allows the solution to penetrate deeper into the dentine and cause further demineralisation. The resulting increase in the depth of demineralisation therefore explains the **back-scattered SEM** observation of our pilot study, indicating that silver $AgNO_3$ particles penetrated deeper and more abundantly into the demineralised dentine than did the SDF solution.

It has been reported that a 0.2 μm $AgNO_3$ solution inhibited mineral induction by the dentine matrix and increased the mineral induction time in a dose-dependent manner.[28] A 25% $AgNO_3$ solution has been used in dentistry for caries control.[2] Although silver ions have antimicrobial properties on biofilm,[29,30] $AgNO_3$ does not favour dentine remineralisation from the perspective of mineral content. However, the situation changes in the presence of fluoride ions. Calcium fluoride (CaF_2) will form instead of soluble calcium nitrate. Some researchers and dentists have used SDF for caries control.[4] In countries such as the US, where SDF is not available, a 5% NaF varnish is used together with a 25% $AgNO_3$ solution.[2] The succinct (unbalanced) chemical reaction between HAP and SDF is as follows.

$$Ca_{10}(PO_4)_6(OH)_2 + Ag(NH_3)_2F \longrightarrow 20\ Ag_3PO_4 + 30\ CaF_2 + NH_4OH$$
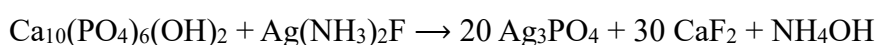
CaF_2 is an important reaction product and acts as a pH-regulated slow-release fluoride reservoir during cariogenic challenges.[1] Our pilot study found that the Ag ions of SDF had not readily penetrated into the demineralised dentine, in either volume or depth, when compared to the $AgNO_3$ solution. This could be because CaF_2 promoted the remineralisation process and the Ag ions therefore diffused less readily into the demineralised dentine. We found the formation of typical clustered granular structures of spherical grains under SEM, suggesting that remineralisation of the demineralised dentine had occurred with SDF application. In addition, this could be one of the main reasons why caries arrested by topical SDF application were reported to have a higher micro-hardness than that of active carious lesions.[31]

An *in vitro* study reported that SDF reacted with the gelatin used as a collagen substitute to produce a CaF_2-like material and metallic silver, but the former dissolved and disappeared after washing with water.[32] In this study, we too could not detect CaF_2 on the SDF-treated dentine surface using XRD. The pH cycling and washing with deionising water between the demineralisation and remineralisation solutions was probably the reason for not detecting CaF_2 in this study.

The organic matrix, consisting primarily of type I collagen, plays an important role in the demineralisation and remineralisation process. The preservation and stability of dentine collagen is fundamental during the remineralisation process, since it acts as a scaffold for mineral deposition.[33] Type I collagen contains approximately 10% HYP by mass, while other proteins contain little or none of this amino acid. HYP is a major component of collagen and

plays key roles in collagen stability. [13, 17] Insoluble collagen molecules in dentine become soluble collagen after dentine degradation, the determination of HYP concentration was used for direct evaluation of degradated collagen in solution. [18] The HAP assay used in current study was a common way for determination of HYP in biological tissue. However, the numerous assay procedures may also indicate the limitations of the method regard to specificity, sensitivity, reproducibility, accuracy and practical approach. [19] It is also noteworthy that the limited repeatability of HYP assay is a concern of some researchers.

Our earlier *in vitro* study demonstrated that 38% SDF possesses a potent inhibitory effect on the activity of MMPs.[12] It is noteworthy that the collagen degradation in this study is produced by bacterial collagenase from *C. histolyticum*. This bacterial collagenase is different from mammalian collagenases, which cleave the collagen at a single site. The bacterial collagenase makes multiple cleavages by hydrolysing the peptide bonds of the amino acid, thus breaking the collagen molecules into small peptides.[18] Therefore, a more powerful degradation of collagen was expected with bacterial collagenase than with mammalian collagenases, making HYP more readily detectable. We found low values of organic matrix degradation (in terms of HYP content), which suggested that SDF and $AgNO_3$ could preserve dentine collagen against bacterial proteolysis. We found that a substantial amount of AgCl precipitated after SDF or $AgNO_3$ application. AgCl is well-known for its antibacterial properties[1,29] and is used in dentistry as an antimicrobial agent in some infection-resistant surgical fabrics or mixed into latex to prevent bacteria from growing on the latex. It is plausible that silver ions interact with a reactive side chain of the bacterial collagenase to inactivate their catalytic functions.[34] However, NaF at an even higher concentration (10%) showed little inhibitory effect on collagen degradation. Since SDF was applied immediately after 96h demineralisation of dentine, it is plausible that SDF interacted with exposed collagen. The interaction between SDF and collagen might contribute to the results of lesion depth and HYP content. Further research can be carried out to investigate whether silver ion can bind and interact with proteins and peptides of collagen, and therefore protect collagen against collagenase challenging.

5. Conclusion

According to the results of this *in vitro* study, 38% SDF could reduce mineral loss and collagen exposure from acid challenge by pH cycling. The use of 38% SDF demonstrated a greater inhibitory effect on dentine demineralisation and collagen degradation than 10% NaF

and 42% $AgNO_3$ solutions, which have equivalent concentrations of fluoride and silver ions, respectively. This comprehensive inhibitory effect enhances the process of dentine caries remineralisation.

Acknowledgements

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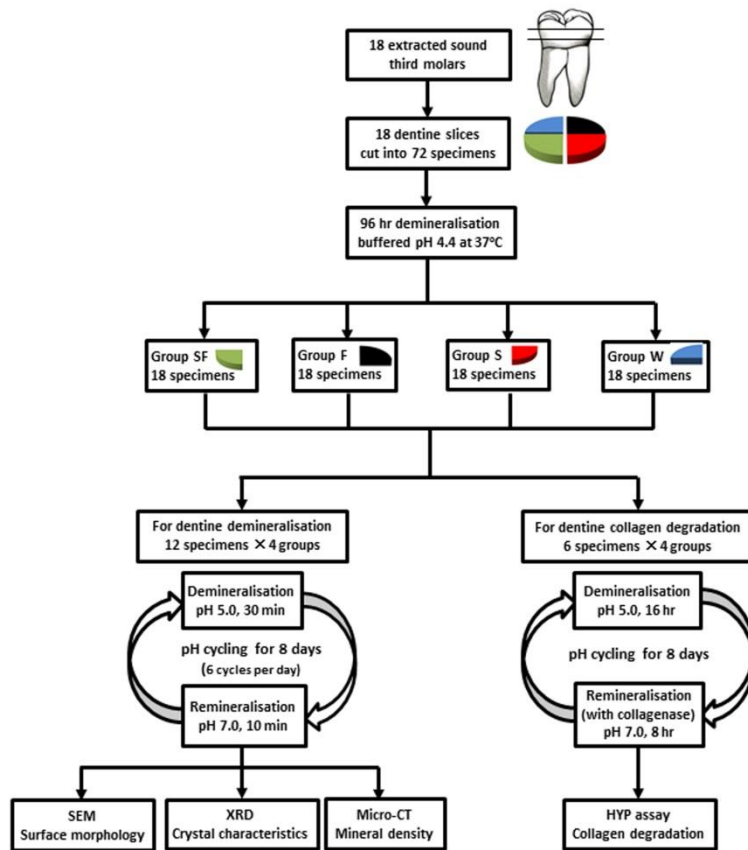# Figure 1 Flowchart of the experiment design

Figure 2 SEM images of surface morphology of artificial dentine caries
(a) 8,000× magnification view of group SF; (b) 30,000× magnification view of group SF;
(c) 8,000× magnification view of group F; (d) 30,000× magnification view of group F;
(e) 8,000× magnification view of group S; (f) 30,000× magnification view of group S; (g)
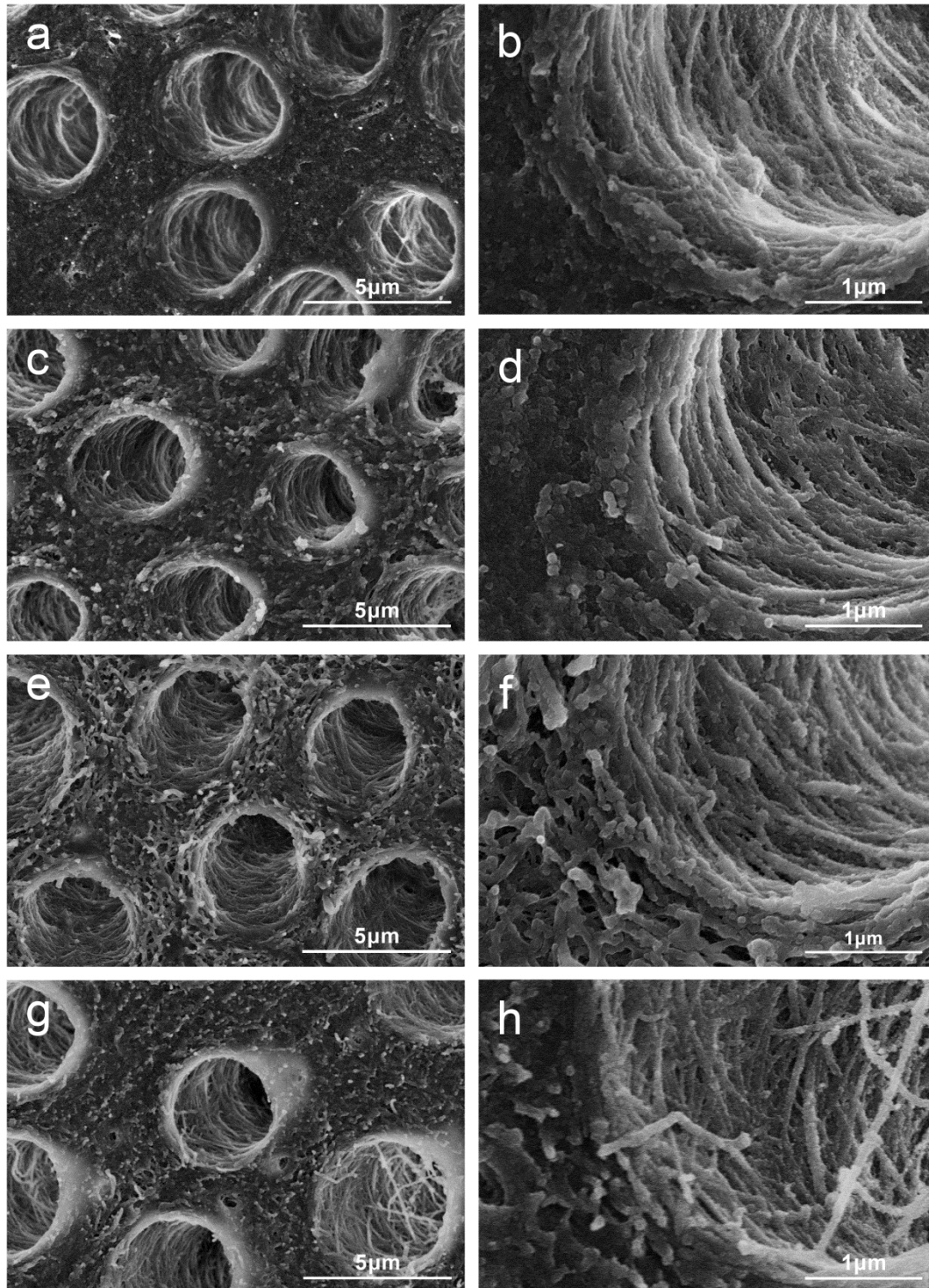8,000× magnification view of group W; (h) 30,000× magnification view of group W.

Figure 3 SEM images of artificial dentine carious lesions (cross-section)
(a) 8,000× magnification view of group SF; (b) 30,000× magnification view of group SF; (c) 8,000× magnification view of group F; (d) 30,000× magnification view of group F; (e) 8,000× magnification view of group S; (f) 30,000× magnification view of group S; (g) 8,000× magnification view of group W; (h) 30,000× magnification view of group W.
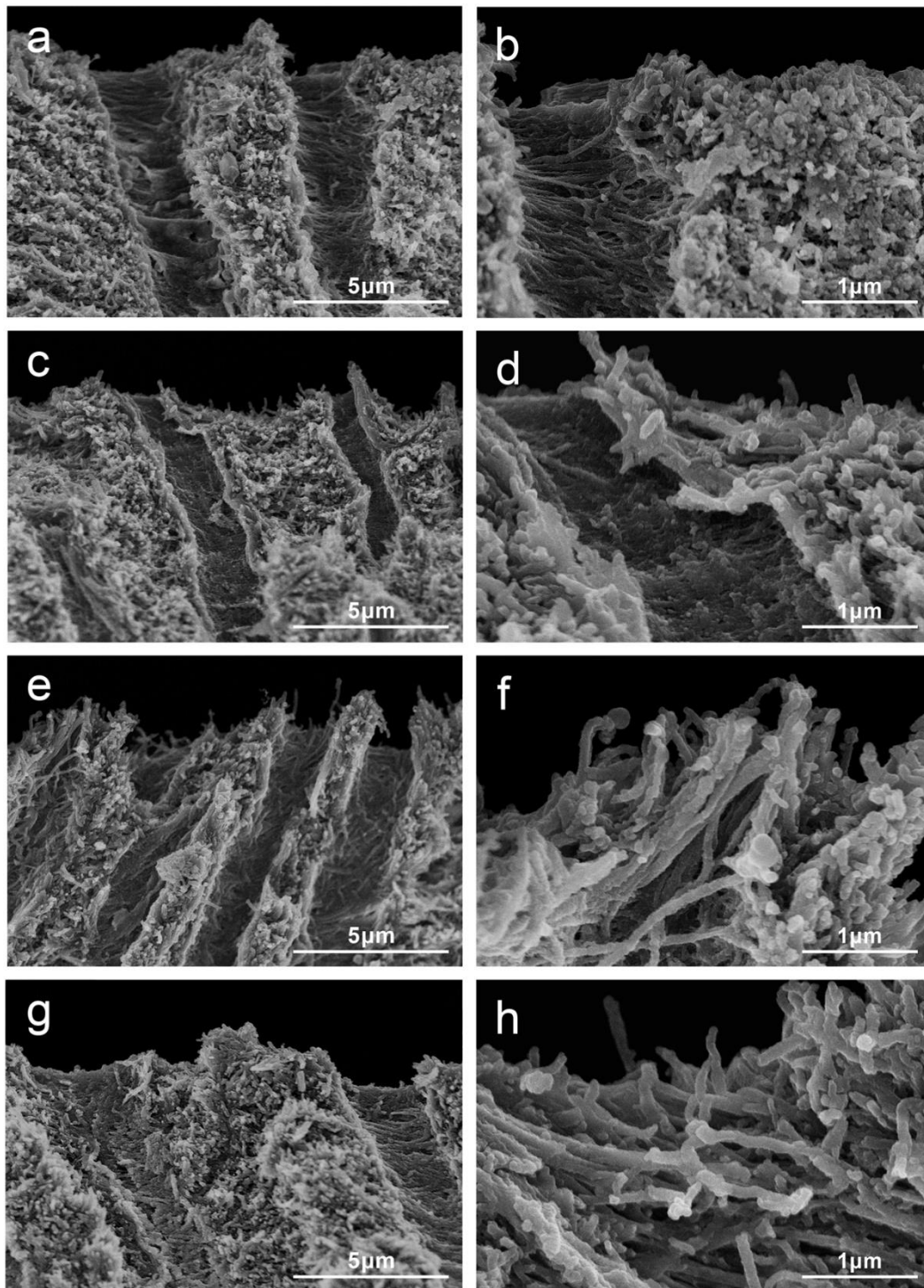
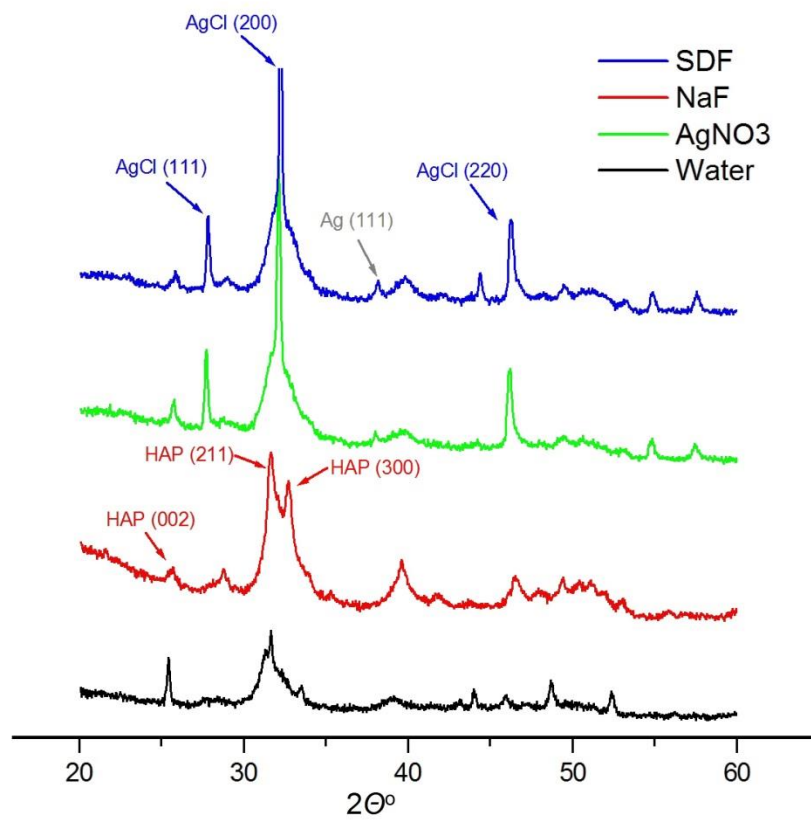# Figure 4 Typical XRD patterns of the four experimental groups

Figure 5 Lesion depths of the four experimental groups
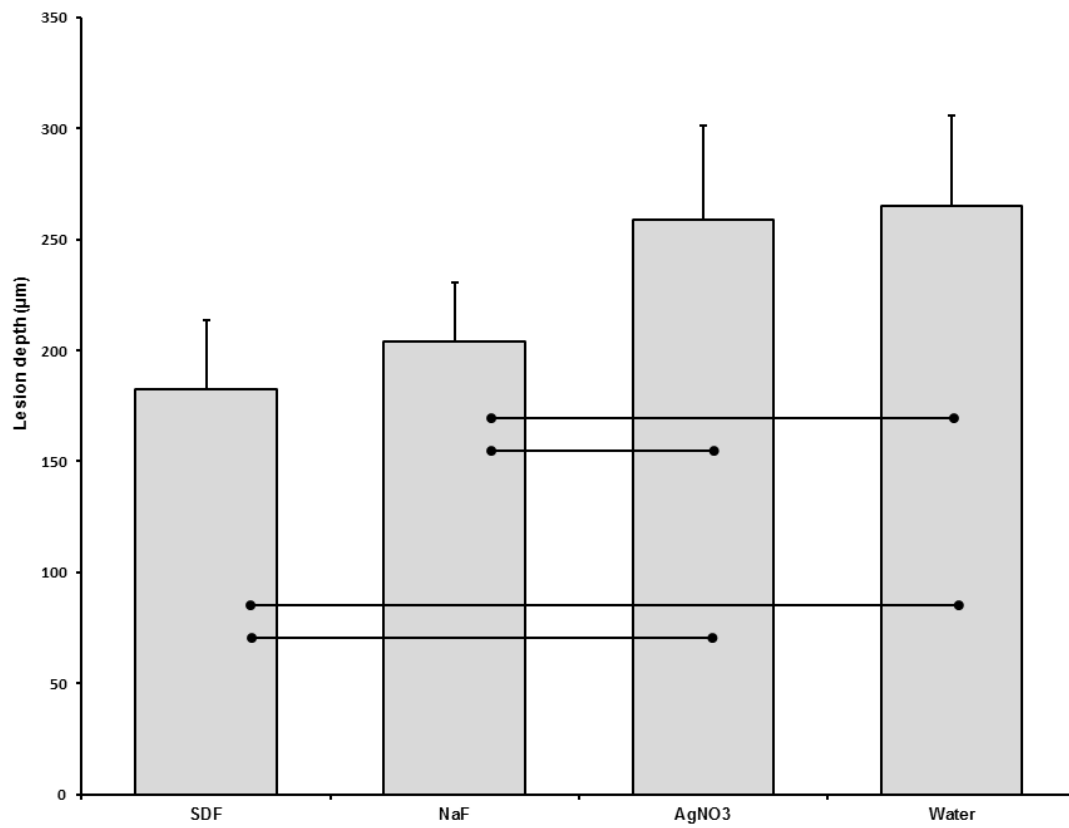Columns linking bars with markers indicate significant differences at p<0.05 between groups.

Figure 6 Hydroxyproline concentrations of the four experimental groups.

Columns linking bars with markers indicate significant differences at p<0.05 between groups.