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An *ex vivo* study of arrested primary teeth caries with silver diamine fluoride therapy

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ABSTRACT

Objectives: This *ex vivo* study compared the physico-chemical structural differences between primary carious teeth biannually treated with silver diamine fluoride (SDF) and carious teeth without such treatment.

Method: Twelve carious primary upper-central incisors were collected from 6-year-old children. Six teeth had arrested caries after 24-month biannual SDF applications and 6 had active caries when there was no topical fluoride treatment. The mineral density, elemental contents, surface morphology, and crystal characteristics were assessed by micro-computed tomography (micro-CT), energy-dispersive X-ray spectrometry (EDX), scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Results: Micro-CT examination revealed a superficial opaque band approximately 150 μm on the arrested cavitated dentinal lesion. This band was limited in the active carious lesion. EDX examination detected a higher intensity of calcium and phosphate of 150 μm in the surface zone than in the inner zone, but this zone was restricted in the active cavitated dentinal lesion. SEM examination indicated that the collagens were protected from being exposed in the arrested cavitated dentinal lesion, but were exposed in the active cavitated dentinal lesion. TEM examination suggested that remineralised hydroxyapatites were well aligned in the arrested cavitated dentinal lesion, while those in the active cavitated dentinal lesion indicated a random apatite arrangement.

Conclusions: A highly remineralised zone rich in calcium and phosphate was found on the arrested cavitated dentinal lesion of primary teeth with an SDF application. The collagens were protected from being exposed in the arrested cavitated dentinal lesion.

Clinical significance: Clinical SDF application positively influences dentine remineralisation.

1. Introduction

Clinical studies have shown that silver diamine fluoride (SDF) prevents and arrests coronal caries in preschool children[1,2] and root caries in elders.[3,4] A review on SDF concluded that it is a safe, effective, efficient, and "equitable" caries control agent that can be used to help meet the World Health Organization Millennium Goals and fulfil the US Institute of Medicine's criteria for 21st-Century medical

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care.[5] SDF has therefore gained its popularity in caries management.[6–8]

Laboratory studies found that SDF has an intense antibacterial effect on cariogenic biofilm[9–11] and possesses a potent inhibitory effect on the activity of matrix metalloproteinases[12] and cysteine cathepsins.[13] SDF treatment can increase the mineral density of enamel carious lesions[14] and the micro-hardness of dentine carious lesions.[15] An *in vitro* study found that principal components of tooth tissue react with SDF and formed calcium fluoride, which has caries-protective effect.[16] Another laboratory study reported SDF could inhibit demineralisation and preserve dentine collagen from degradation in demineralised dentine.[17] These laboratory studies used different *in vitro* models that simulated certain factors of clinical conditions, but they are by no means an apt representation of the sophisticated environment in the oral cavity. Therefore, we investigated exfoliated primary teeth from children in this study, the condition of real mouth is much more complex than laboratory models and results could directly reflect the clinical situation.

Early childhood caries are prevalent among many of the preschool children in Hong Kong.[18] In this study, mobile-carious upper-central incisors that would soon be exfoliated were collected from children who had been enrolled in a 24-month randomized clinical trial with parental consent. The clinical trial compared the caries-arresting effects on children receiving biannual SDF application. Incisors with active caries from children who did not accept the black staining and did not join the clinical trial were collected for comparison. The purpose of this *ex vivo* study was to compare the physico-chemical structural differences between primary carious teeth biannually treated with SDF and carious teeth without such treatment. The null hypothesis of the study is there is no difference in physico-chemical structure between primary carious teeth biannually treated with SDF and carious teeth without such treatment.

2. Materials and methods

2.1. Collection of teeth

This *ex vivo* study is associated with a 24-month randomized clinical trial approved by the University of Hong Kong/Hospital Authority Hong Kong West Cluster Institutional Review Board (IRB UW08-052). The clinical trial compares the effectiveness of biannual SDF application on arresting caries treatment on primary teeth. Generally healthy 4-year-old children who had active caries (cavitated dentinal lesions) on primary teeth attending the first year of the selected kindergartens in Hong Kong were recruited. Ninety-eight participating children were assigned to receive biannual topical applications of 38% SDF (Saforide-38%, Toyo Seiyaku Kasei, Japan). Their carious teeth were dried and gently cleaned with gauze, and SDF solution was then applied with a disposable microbrush. Treatment was considered successful if the tooth was asymptomatic, no sign of pulpal pathology (absence, sinus tract, discoloured tooth, or hypermobility) and the cavitated dentinal lesion was hard on probing (arrested caries) at follow-up clinical examination.

At the end of the 24-month study, six 6-year-old children who had mobile-unfilled arrested carious primary upper central incisors receiving biannual SDF applications were invited to participate in this study. The generally healthy 6-year-old children who had no professional topical fluoride treatment of the same selected kindergartens and did not join the clinical trial were also examined. Six children who had mobile carious upper central incisors which would soon be exfoliated were invited to join this *ex vivo* study. These 6 incisors, which had active caries not involving the dental pulp, were extracted with parental consent. Hence, six primary upper central incisors with arrested cavitated dentinal lesions treated with biannual SDF and 6 incisors with cavitated dentinal lesions with no topical fluoride application were collected. They were fixed in 10% neutral formalin solution and stored at 4 °C before laboratory investigation.[15]

2.2. Assessment of mineral density

The 12 collected incisors were scanned by a compact X-ray micro-CT scanner (SkyScan 1076, SkyScan Company, Antwerp, Belgium) to assess the mineral density of the cavitated dentinal lesions. The X-ray source was operated at a voltage of 100 kV and a current of 80 lA. The highest spatial resolution of 9 lm was used for the scanning. Signal-to-noise ratio was chosen at 5, and a 1 mm aluminium filter was used to take away the softest X-rays.

Scanning results of each tooth were reconstructed using the reconstruction software NRecon (SkyScan Company, Antwerp, Belgium). The reconstructed 3-D images were viewed and processed using the data-analyzing software CTAn (SkyScan Company, Antwerp, Belgium). Cross-sectional images in each tooth were located from the reconstructed 3-D image of each specimen 9]. Typical images with a 500 μm line in length were randomly selected below the surface of the tooth from the centre of the lesion towards the pulp; the lines were analyzed by special-image analysis software (ImageJ, National Institutes of Health, USA) in terms of grey value.

2.3. Elemental analysis

Eight teeth (4 teeth with arrested carious lesions and 4 teeth with active carious lesions) were embedded in acrylic resin and sectioned along the long axis of the tooth into 2 halves using a copper cutting disc.[15] The exposed cross-section surfaces of the tooth block were directly (without polish) treated with 1% acetic acid for 5 s and ultrasonically washed with deionised water to remove the smear layer. One-half of the tooth block was used for elemental analysis. Lines with 500 μm in length were randomly selected below the surface of the tooth from the centre of the dentinal lesion towards the pulp; the lines were analyzed by line-scan in terms of calcium, phosphorus, fluoride, and silver ion levels *via* energy-dispersive X-ray spectroscopy (EDX) under scanning electron microscopy (SEM) (Hitachi S-4800 FEG Scanning Electron Microscope, Hitachi Ltd., Tokyo, Japan).

2.4. *Evaluation of surface morphology*

The other half of the tooth block was then dehydrated in a series of ethanol solutions, critical-point dried in a desiccator, and sputter-coated with carbon. The surface morphologies of the specimens were evaluated under SEM (Hitachi S-4800 FEG Scanning Electron Microscope, Hitachi Ltd., Tokyo, Japan) at 5 kV in high-vacuum mode.

2.5. *Study of crystal characteristics*

Four teeth (2 with arrested caries and 2 with active caries) were used for the study of crystal characteristics. The surface layers of the arrested dentinal lesions or the active dentinal lesions were scraped by a blade. The fine particles were collected and stained with 1% phosphotungstic acid to examine the status of the collagen. The powder was then dispersed in dehydrated ethanol to minimize the dry artefacts caused by the vacuum environment of the transmission electron microscope (TEM). Next, the power was applied to the grids by dipping. Subsequently, the specimens were observed by TEM (FRI Tecnai G2 20 TEM, FEI, Eindhoven, Netherlands) with electron diffraction (INCA X-sight, Oxford Instruments, High Wycombe, UK). Selected area electron diffraction (SAED) was used to identify crystal structures and examine crystal defects.

3. Results

A typical mineral-density profile with micro-CT image along the assessed lesion depth (white line in Fig. 1a) in the arrested dentinal lesion and the active dentinal lesion of the tooth specimens is shown in Fig. 1. A distinct opaque and dense layer approximately 150 μm was found on the surface of the arrested dentinal lesion. The grey value of this layer was higher than that of unaffected dentine in the inner part of the tooth (Fig. 1b). The mineral density of the active dentinal lesion was considerably lower than that of unaffected dentine, although there was a surface layer that was relatively denser than that of the adjacent inner zone of the body of the lesion (Fig. 1c and d).

The EDX results were consistent with the typical mineral-density profile. The intensity of both calcium and phosphorus in the outermost 150 μm of the arrested dentinal lesion was higher than that found in the lesion body, and it was even higher than that of the unaffected dentine in the inner part of the tooth (Fig. 2a and b). The intensity of silver and fluoride

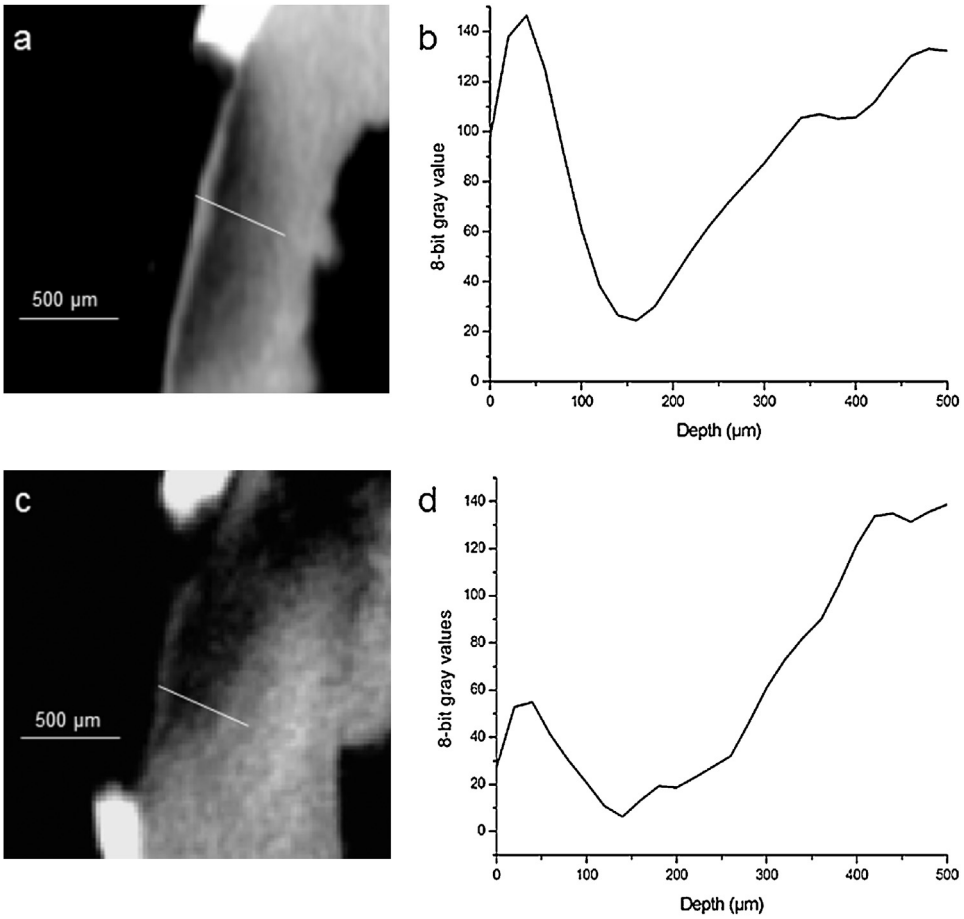

Fig. 1 – Micro-CT images and grey-value profiles of the dentine carious lesions. (a) Cross-sectional micro-CT image of the arrested carious lesion; (b) grey-value profile along the path (white line in a); (c) cross-sectional micro-CT image of the active carious lesion; (d) grey-value profile along the path (white line in c).

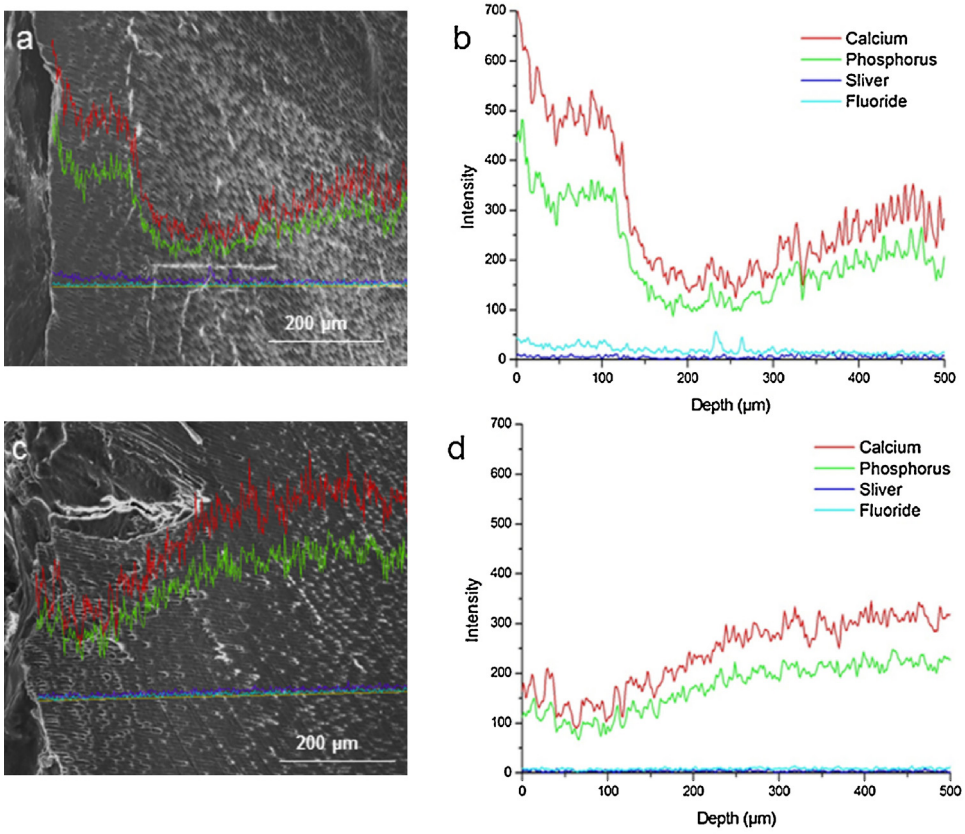

Fig. 2 – Line-scan images of the dentine carious lesions. (a) Cross-sectional image of the arrested carious lesion; (b) line-scan elemental profile (calcium, phosphorus, fluoride, and silver) along the path (line in a); (c) cross-sectional image of the active carious lesion; (d) line-scan elemental profile (calcium, phosphorus, fluoride, and silver) along the path (line in c).

was relatively low for the arrested dentinal lesion, but was inconspicuous for the active dentinal lesion. The intensity of calcium and phosphorus in the active carious lesion was lower than those in the unaffected dentine (Fig. 2c and d).

The surface morphology of the arrested dentinal lesion under SEM showed a relatively smooth surface with few dentine collagen fibres exposed (Fig. 3a). Dense granular structures of spherical grains were found in the inter-tubular area at high magnification (Fig. 3b). The surface morphology of the active dentinal lesion was porous and rough (Fig. 3c). Collagens were found to be exposed, disorganized, and sparsely distributed at high magnification (Fig. 3d).

Needle-shaped crystallites could be seen (open arrow) under TEM in the fine particles collected from the surface of the arrested dentinal lesion (Fig. 4a). Some nano-particles (diamond arrow) were confirmed to be silver by EDX (Fig. 4a). Lattice spacing and intersection characteristics of hydroxyapatite could be identified at high magnification (Fig. 4b). SAED showed characteristics of the minor [0 0 2], [1 1 2] and major [2 1 1] planes of crystalline apatites. The arc-shaped SAED patterns ascribed to the [0 0 2] plane of apatite suggested that the c-axes of the hydroxyapatite platelets had a preferential orientation (Fig. 4c).

In contrast, crystallites could barely be recognized in the active dentinal lesion (Fig. 4d); only rounded crystallites were found at high magnification (Fig. 4e). The crystallites were generally smaller than those observed in the arrested dentinal lesion (Fig. 4b and e). SAED of these structures were devoid of arc-shaped patterns, suggesting a more random crystallite arrangement (Fig. 4f).

4. Discussion

In this *ex vivo* study, primary anterior central incisors with arrested dentinal lesions were collected from 6-year-old Chinese kindergarten children receiving a topical application of 38% SDF every 6 months for 24 months. Primary incisors with active caries that received no professional topical fluoride therapy were used for comparison. We did not use a split-mouth method to evaluate the test and control samples, because the study design is not accepted in clinical trials of topical fluoride therapy due to the crossover effect. Our previous 30 months clinical research indicated there was no evidence to show that removal of carious tissues prior to application of the SDF has an effect on their ability to arrest dentine caries ($p > 0.05$).[1] Thus, the carious cavity was cleaned by gauze without removal of debris and soften dentine before SDF application. This could make the application process easier and faster, in particular for child patients.

These teeth were collected from children from the same selected kindergartens. All the children were 6 years old, and

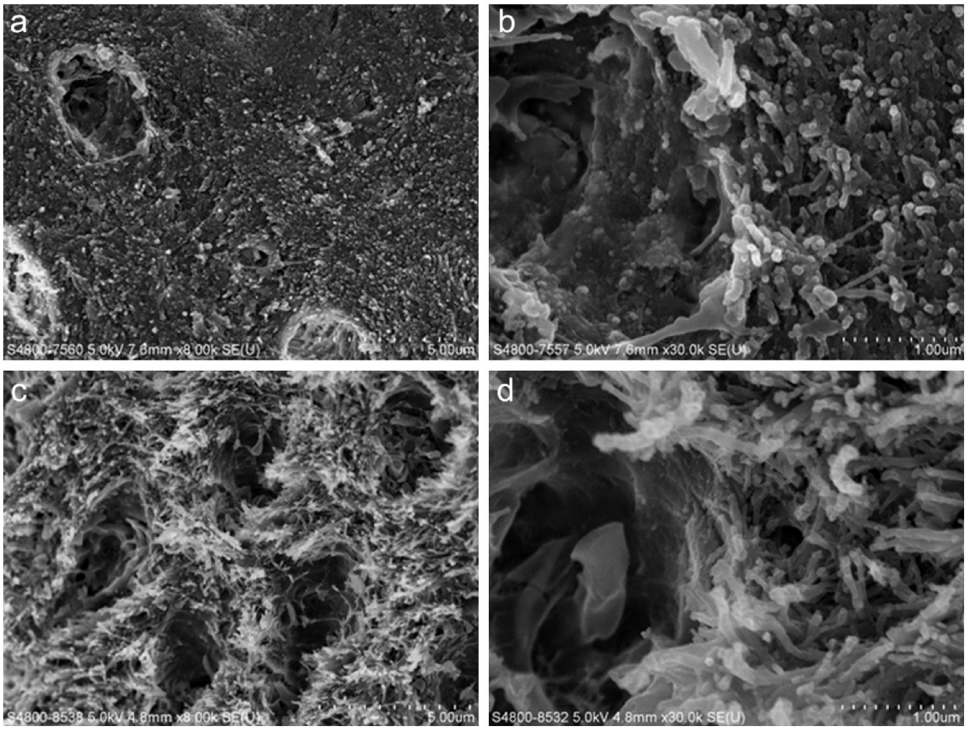

Fig. 3 – SEM images of the dentine carious lesions. (a) 8000× magnification view of arrested carious lesion; (b) 30,000× magnification view of arrested carious lesion; (c) 8000× magnification view of active carious lesion; (d) 30,000× magnification view of active carious lesion.

had similar caries experience and oral hygiene practices. Therefore, their general caries risks should be similar. In this study, it is a challenge for us to collect mobile, unfilled upper central incisors with no pulp pathology and were expected to exfoliate soon from children who had parental consent for extraction. In addition, teeth that exfoliated between treatments could not be collected. With these circumstances, we were only able to collected 6 deciduous primary teeth from each group.

It is essential to note that the children used fluoridated toothpaste. The water supply in Hong Kong is also fluoridated at 0.5 ppm.[19] It is not known how this level of fluoride exposure would affect the physico-chemical structural properties of the carious teeth. It is desirable to collect arrested carious teeth that were not treated with SDF and health teeth for comparison. This information would be pertinent, but we could not find arrested carious incisors and health incisors among the examined children.

This study investigated the mineral and organic properties of the arrested- and active-dentinal lesions on the primary upper-central incisor, which is one of the most common teeth to suffer from dental decay.[18] Given that the dentine carious lesions varied in size and could be at different stages in progression when they received the topical SDF application, the mineral content and the lesion depth could vary appreciably among the specimens. We therefore selected typical results for presentation; the analyses were qualitative and the findings of this study may not be generalized. However, the samples in the study can be examined in detail and in depth to yield relevant results to understand the mechanism of SDF in arresting caries. The inherent disadvantage of SDF application is using SDF to arrest caries is that the lesions will be stained black. It was suggested that $AgPO_4$ was formed and it is readily turns black under the influence of reducing agents.[6]

According to the results of this study, the null hypothesis was rejected. The mineral density of the outermost layer of the active carious lesion was found to be higher than that in the body of the lesion. This increased mineral content in the surface layer and the formation of a zone of higher mineral content within the body of the lesion on a demineralised dentine lesion was reported by a previous *in situ* study.[20] The availability of fluoride in drinking water and fluoride toothpaste would promote remineralisation in the presence of calcium and phosphate ions from saliva. In addition, the teeth with a vital pulpo-dentinal organ responded to most exogenous stimuli through the apposition of minerals along and within the dentinal tubules.[21] This relatively denser layer was less dense than that in unaffected dentine; it should be considered demineralised tissue rather than remineralisation.

Interestingly, a distinct and dense layer was found in the arrested dentinal lesion, and the density of this layer was higher than that of unaffected dentine in the inner part of the tooth. Even the dentine carious-lesion depth varied among different specimens; the width of this dense surface layer was approximately 150 μm and a high mineral content of calcium and phosphorus was found in this layer. This finding corroborated our previous study that reported surface micro-hardness of the surface layer of the arrested caries

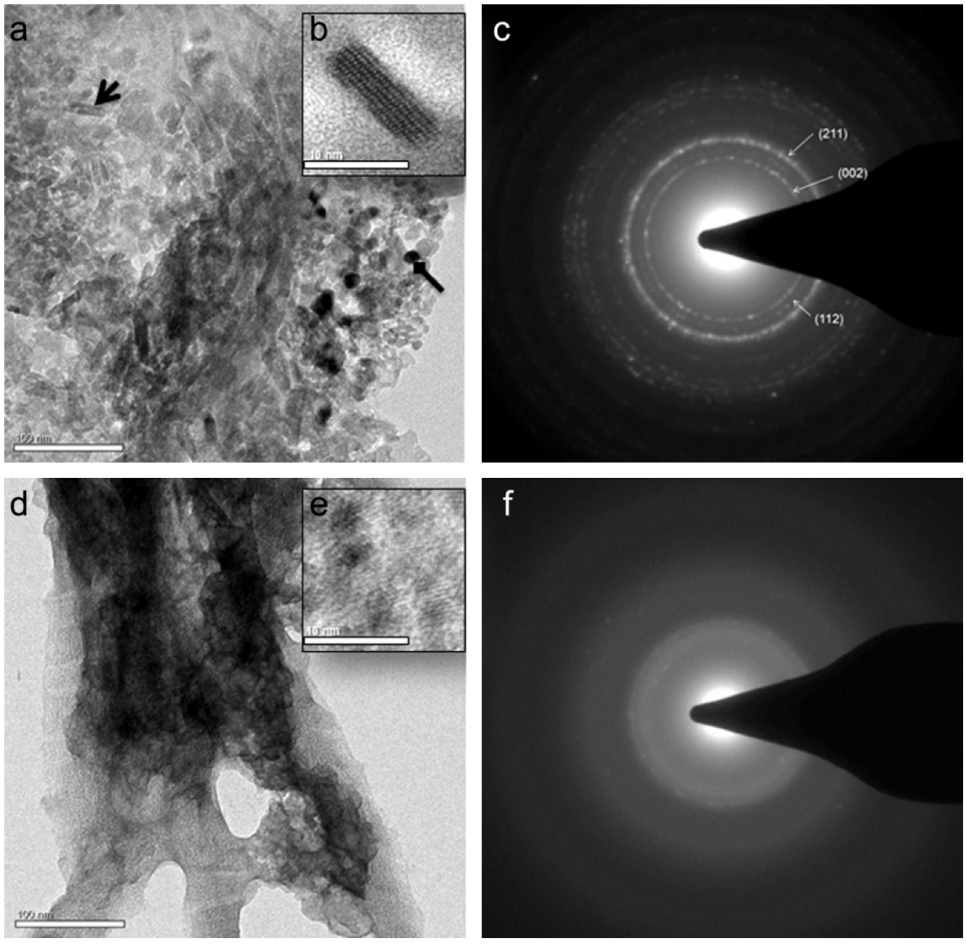

Fig. 4 – TEM and SAED images of the dentine carious lesions. (a) TEM of fine particles collected from the surface of the arrested carious lesion; (b) high magnification view of the crystallite in (a); (c) typical SEAD pattern of arrested carious lesion; (d) TEM of fine particles collected from the surface of the active carious lesion; (e) high magnification view of the crystallite in (d); (f) typical SEAD pattern of active carious lesion.

after fluoride applications was higher than that of inner unaffected sound dentine. [15]

Fluoride treatments on dentine reduce its solubility, but the fluoride level required to inhibit demineralisation is around 10 times higher than that for enamel.[22] A 38% SDF solution contains 44,800 ppm fluoride,[17] and this high fluoride concentration is favourable in inhibiting dentine demineralisation. In this study, typical clustered granular structures of spherical grains with higher contents of calcium and phosphorus were found in the SDF-treated lesion surface, suggesting there was remineralisation. Calcium fluoride-like globules presumably formed after topical fluoride therapy.[22] Calcium fluoride can act as a temporary storage of fluoride, which can gradually release to promote remineralisation.

In this study, the amount of fluoride in the arrested dentinal lesions was not high. This finding concurs with a previous laboratory study on the reaction of SDF with hydroxyapatite. The laboratory found the fluoride content became low after washing with water.[22] Our previous laboratory pH cycling study also could not find calcium fluoride on SDF-treated demineralised dentine.[23] Lambrou et al. suggested that fluoride can be dissolved, resulting in the increase of fluoride concentration in saliva within 12 h after the topical fluoride application.[24] In this study, SDF was applied biannually to the dentine caries of the primary teeth. Some calcium fluoride might remain in the carious lesion, and the amount should be below the threshold of detection by EDX.[22] This could explain why fluoride could barely be observed in the remineralisation zone of the lesion.

Collagens were exposed noticeably in the active dentinal lesion in default of the hydroxyapatite structure, suggesting dentine demineralisation. Collagen is the structural backbone of dentine, which holds together the apatite crystallites.[25] However, a dense granular structure of spherical grains was observed under SEM on the cross-section surface in the arrested carious lesion, which indicated extra-fibrillar mineral formation.[26] The collagens were therefore protected from being exposed. SDF inhibits the activity of matrix metalloproteinases,[12] cysteine cathepsins,[13] and bacterial collagenase.[23] Therefore, SDF might be able to protect the collagens from destruction by these endopeptidases and collagenase, which break fibrillar collagens into distinctive fragments.[27] In

addition, the high concentration of silver ion in SDF could influence physio-chemical properties of collagen structure or morphology, and collagen might be modified to be more resistant structure against collagens attaches.[13,23] The sound collagens are the bases for calcium and phosphate to precipitate and for hydroxyapatite to form. In this study, the sizes of the hydroxyapatite crystallites were small and randomly arranged in the active carious lesion. Nevertheless, the crystallites were well-aligned and organized in the arrested dentine carious lesion and their sizes were generally larger than that in the active carious lesion.

A 38% SDF solution is strongly alkaline. It might provide an alkaline environment that facilitates the formation of covalent bonds between the phosphate groups on proteins, thus providing a favourable setting for crystallites to grow. This is a mechanism through which phosphate in saliva was attached to dentine collagen. Once the phosphate built into the collagen, it contributed to the binding sites for calcium ions, thereby facilitating the apatite nucleation onto the collagen.[25]

A laboratory study demonstrated that metallic silver particles were formed when hydroxyapatite powder reacted with SDF.[16] In this study, SEM with back-scattered signal and high magnification showed that nanoscopic highlight particles were found in the collagens in arrested-dentinal carious lesions (data not shown). TEM-EDX analysis indicated those nanoscopic particles formed in the remineralised layer were metallic silver. However, SEM-EDX results did not show the presence of silver, this might because the total amount of silver ion in the lesion was small after 6 months of application. In addition, silver ions were in nano-size and disperse, which is also hard to be detected by low magnification SEM-EDX.[8,16] Silver nano-particles were shown to have a great inhibitory effect on growth of cariogenic bacteria such as *Streptococci mutans* and *Streptococci sanguis*.[28] Nano-particles can break through the permeability of the outer membrane of the bacteria cell and inactivate respiratory chain dehydrogenase.[29] This potent anti-microbial effect of nanoscopic silver might be one of the major reasons why caries can be arrested by SDF even without their removal.[15]

5. Conclusion

In this study, a dense, highly mineralized surface layer was formed on the arrested cavitated dentinal lesion of primary teeth after a 24-month biannual 38% SDF application. The mineral content of calcium and phosphate was found to be higher than that of the inner unaffected dentine. Compared with those in the active cavitated dentinal lesion, the crystallites on the arrested cavitated dentinal lesion were well-aligned and more organized. Moreover, the collagens were protected from being exposed in the arrested lesion, but were exposed in the active lesion.

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