Prevention of dentine caries using silver diamine fluoride application followed by Er:YAG laser irradiation: an *in vitro* study

Short title: Caries prevention by laser and silver diamine fluoride

May L. Mei, BDS, MDS, PhD

Leticia Ito, BDS

C.H. Chu, BDS, MDS, PhD

Edward C.M. Lo, BDS, MDS, PhD

C.F. Zhang, BDS, MDS, PhD

Faculty of Dentistry, The University of Hong Kong, Hong Kong

Key words *: laser, preventive, silver diamine fluoride, caries, nano-indentation, micro-hardness*

Correspondence to : Dr C. H. Chu
Faculty of Dentistry
The University of Hong Kong
34 Hospital Road
Hong Kong SAR, China

Tel: +852 2859 0287
Fax: +852 2858 7874
Email: chchu@hku.hk

Abstract

Objective: *To evaluate the preventive effect of Er:YAG laser (EYL) irradiation followed by silver diamine fluoride (SDF) application on dentine with cariogenic biofilm challenge.*

Methods: *Twenty-four dentine slices were prepared from extracted sound human third molars. Each slice was cut into four parts for SDF application followed by EYL irradiation (group SL), SDF application (group S), EYL irradiation (group L) and water (group W). The specimens were subjected to cariogenic biofilm challenge for 12 hours, followed by immersion in a buffered remineralising solution containing calcium chloride and sodium hypophosphate for 12 hours at 37^{o}C. Surface morphological changes in the specimens were examined using scanning electronic microscopy. Elemental analysis was performed using energy dispersive X-ray spectrometry. Micro-mechanical properties were investigated by nano-indentation.*

Results: *The specimen surfaces of groups SL and L showed laser melting contours with narrowed dentinal orifices. Group S showed a partial tubular occlusion. A porous surface was observed in group W, indicating demineralisation. The mean (SD) fluoride weight percentages were 3.93 (0.91), 3.10 (0.61), 0.17 (0.09) and 0.32 (0.07) in groups SL, S, L and W, respectively ($p<0.001$; SL, S>L, W). The mean (SD) micro-hardness values in GPa were 1.84 (0.22), 0.49 (0.13), 0.41 (0.11) and 0.30 (0.06) in groups SL, S, L and W, respectively ($p<0.001$; SL>S>L, W). The mean (SD) elastic moduli in GPa were 75.1 (7.2), 20.0 (1.3), 24.3 (5.2) and 20.2 (2.8) in groups SL, S, L and W, respectively ($p<0.001$; SL>S, L, W).*

Conclusion: *SDF application followed by EYL irradiation on a dentine surface increased its resistance to cariogenic biofilm challenge.*

Introduction

Clinical trials have found that silver diamine fluoride (SDF) is effective at arresting coronal [1,2] and root dentine caries [3]. *In vitro* studies have reported that SDF can inhibit biofilm formation [4] and matrix metalloproteinase activities [5]. SDF also hardens the caries lesion, reduces loss of calcium and phosphate ions and lessens collagen damage [6,4]. SDF has thus gained popularity in dentistry. One review of 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's Millennium Goals and fulfil the US Institute of Medicine's criteria for 21st century medical care [7].

Laser is also becoming common in clinical dental care and is one of the promising new modalities used for caries management. Laser has been shown to increase fluoride uptake in root [8,9] and enamel surfaces [10]. Studies have also demonstrated that a combined fluoride-laser treatment makes enamel more resistant to acid than do either laser or fluoride treatments alone [11,12,9]. Both enamel and dentine take on important roles in carious progression. Dentine also needs to be protected from caries on exposed root surfaces and during post-operative procedures. However, little dentine-related mechanistic investigation into fluoride-laser treatment has been conducted. It is also noteworthy that most studies have simply been *in vitro* studies performed in the absence of oral biofilm. In fact, oral biofilms, particularly cariogenic bacteria, play a crucial role in caries development and progression.

Various types of laser related to the prevention of dental caries have been documented over the past several years [13]. Er:YAG laser has a wavelength of 2,940 nm and is strongly absorbed by water. It is thus effective and efficient in dental hard tissue ablation. Er:YAG laser has been studied in endodontic [13], periodontic [14], restorative [15] and surgical treatments [16]. A great advantage of Er:YAG is that it has little chance of pulpal damage if used under sufficient water cooling. Minimal pain has been reported with its use, and it is thus used without local anaesthesia. Er:YAG laser can also transform enamel hydroxyapatite into fluoridated hydroxyapatite to reduce enamel solubility as a preventive treatment for enamel caries [11]. This *in vitro* study aimed to evaluate the preventive effect of Er:YAG laser irradiation followed by SDF application on dentine surfaces with cariogenic biofilm challenge, an area that has not yet been the subject of any study.

Materials and methods

This study was approved by the Institutional Review Board (IRB UW08-052). A flow chart of the study is given in Figure 1. Extracted sound human molars were collected with patient consent. Twenty-four dentine slices with thicknesses of 3 mm were prepared from the molars. The surfaces of the slices were polished using micro-fine 4,000 grid sanding paper. The polished slices were examined using a stereomicroscope to exclude samples with cracks, hypoplasia or white spot lesions. The examined slices were treated with 1% citric acid for 5 min and ultrasonically washed with deionised water to eliminate the smear layer. After autoclaving, each slice was cut into four parts for different treatments. In the first group (group SL), the specimens underwent a topical application of a 38% SDF solution (Saforide; Toyo Seiyaku Kasei Co. Ltd., Osaka, Japan) with a microbrush (Micro applicator – regular, Premium Plus International Ltd., Hong Kong, China). After a 20 min treatment, the excess SDF solution on the slice surface was carefully wiped off with a sterilised dry cotton bud. Subablative low-energy Er:YAG laser irradiation was applied to the specimen surface at a VSP (very short pulse, 100 μs) mode setting of 100 mJ and a 20 Hz repetition rate (output power 2 W) for 1 sec/mm^2 (Fidelis Plus III, Fotona, Slovenia, EU). In the second group (group S), the specimens were topically treated with a 38% SDF solution. In the third group (group L), the specimens were treated with Er:YAG laser irradiation. Specimens in the fourth group (group W) received deionised water application as the control.

Biofilm challenge

Five species of bacteria, namely *Actinomyces naeslundii* ATCC 12014, *Lactobacillus acidophilus* ATCC 9224, *Lactobacillus rhamnosus* ATCC 10863, *Streptococcus mutans* ATCC 35668 and *Streptococcus sobrinus* ATCC 33478, were used to develop a five-species cariogenic biofilm [4]. A single colony was picked from each culture plate to prepare 24-hour broth cultures in a brain heart infusion (BHI) broth at 37^{o}C under anaerobic conditions. After centrifugation, cell pellets were harvested and washed twice with phosphate buffered saline (PBS) [4]. Bacterial suspensions were then prepared in a BHI broth to a cell density of McFarland 4 (10^9 cells/mL). Afterwards, 300 μl aliquot of each bacterial suspension was inoculated on each specimen. Each specimen was then put into 1 ml of a 5% sucrose solution in one well of a 24-well plate and incubated anaerobically at 37^{o}C for 12 hours.

Remineralisation

After the biofilm challenge, the specimens were rinsed with deionised water and immersed into an ultrasonic bath for 10 min to remove the biofilm. Twelve specimens were then taken out for assessment. The remaining 12 specimens were immersed into a buffered (pH 7) remineralising solution containing 1.5 mM Ca $(NO_3)_2$, 0.9 mM NaH_2PO_4 and 0.15 M KCl at 37°C for 12 hours before evaluation.

Surface morphology assessment

Scanning electron microscopy (SEM) was used to study the surface morphologies of four specimens in each group (Hitachi S-4800 FEG Scanning Electron Microscope, Hitachi Ltd., Tokyo, Japan). In preparation for SEM, the specimens were rinsed in 4% (vol/vol) formaldehyde followed by 1% (vol/vol) PBS. They were then soaked in a 1% osmium tetroxide solution for 60 min, washed in distilled water, dehydrated in a series of ethanol solutions, dried in a desiccator and sputter-coated with gold. The surface topographies of the dentine surfaces were examined under SEM at 20 kV in high-vacuum mode.

Chemical analysis (elemental assessment)

Eight specimens from each group were sectioned vertically for an elemental assessment of the specimens in the cross-section. The levels of calcium (Ca), phosphorus (P), fluoride (F) and silver (Ag) ions in the dentine surfaces were analysed by energy-dispersive X-ray spectroscopy (EDS) under SEM. An elemental assessment was performed by measuring five 5×5 μm^2 square areas under the dentine surface in each sample, and the mean weight percentages of Ca, P, F, Ag and the Ca/P ratio were calculated [4].

Mechanical analysis (nano-indentation assessment)

The micro-hardness values of the dentine slice surfaces were assessed by a pyramidal diamond tip with a diameter of 20 nm secured to a nano-indenter (Nano Indenter G200, Agilent Technologies, Inc., CA, USA) at room temperature using Aglient NanoSuite 6.1 Professional software (Agilent Technologies, Inc., CA, USA). The samples were dehydrated in a serial of ethanol before testing. The force for the indentation was 100 mN, and 40 indentations were

performed on each specimen. The micro-hardness and elastic modulus (Young's modulus) of the specimens were calculated and the load-displacement curve was recorded.

Statistical analysis

All of the data were assessed for a normal distribution using the Shapiro-Wilk test for normality ($p>0.05$). One-way ANOVA was used to compare the weight percentages of Ca, P, F, Ag, the Ca/P ratios, the micro-hardness values and the elastic moduli between 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 of significance was taken as 5% for all of the analyses.

Results

The dentine surface morphology of the specimens from the same slice with different treatments is shown in Figure 2. Consistent surface morphologies were observed in the four examined specimens in all of the treatment groups, with or without remineralisation. The surfaces of groups SL and L showed irregular, laser-induced explosive contours. A partial occlusion of dentinal tubules was found in all four treatment groups after immersion in the remineralising solution. The dentine surface of group W was more porous than its counterparts in other treatment groups.

The elemental assessment results are shown in Table 1. The F content of the specimens increased in all of the treatment groups after remineralisation compared with after biofilm challenge. In addition, the F content in group SL was higher than in the other three groups, and the F content in group S was higher than in groups L and W ($p<0.001$). The Ag content was relatively stable in groups S and SL before and after remineralisation. The Ca and P contents in group SL were higher than in group W while no difference was detected in the Ca/P ratios after remineralisation.

The nano-indentation results are shown in Table 2. Both the micro-hardness and elastic moduli increased substantially in group SL after remineralisation ($p<0.01$). The load-displacement curves (Figure 3) demonstrate that the nano-indenter penetrated deeper into the dentine surface of

the specimens in groups S, L and W compared with those in group SL, particularly after remineralisation.

Discussion

This study aimed to develop a superficial demineralisation of dentine with cariogenic biofilm challenge of about 70 μm. The elemental content and micro-mechanical properties of the dentine surfaces of specimens were thus measured. The results demonstrate the profound caries-preventative effect of Er:YAG laser irradiation followed by SDF application on dentine surfaces with cariogenic biofilm challenge. Previous fluoride/Er:YAG laser studies have reported the effects on dental hard tissues directly after fluoride-laser application [13,17], with some subjecting their enamel or dentine samples to acid challenges using chemical models such as the pH-cycling model [11,18,9]. A chemical model uses an artificial acidic environment to demineralise teeth. Bacterial biofilm is involved in the dental caries, causing the demineralisation of dental hard tissues. Thus, this study generated caries-like lesions on its dentine specimens using the bacterial biofilm model to simulate a more clinically relevant setting [4]. This method reproduced some of the characteristics attributed to natural dentine caries, including a widespread demineralisation of intertubular dentine, collagen fibrils depletion and the discreet opening of dentinal tubules [19,20].

Er:YAG laser has a wavelength of 2,940 nm, which exactly matches the maximal absorption in water and is about 15 times higher than the absorption of carbon dioxide laser [21]. A low-watt (2 W) and very short pulse (100 μs) subablative Er:YAG laser irradiation was chosen because demonstrable ablation can be observed at a power of around 6 W in dentine [21]. The energy of Er:YAG laser is mainly absorbed by the water molecules present in the organic and mineral contents, and this overheats the water molecule by raising its kinetic energy, causing micro-explosions of the hard tissue. In this study, the heating effects were minimised using sharp rectangular pulses over a short time duration. However, irregular, laser-induced explosive contours were observed on the dentine surfaces after irradiation. This observation concurred with the findings of Hossain et al. [22]. One *in vitro* study found that Er:YAG laser irradiation demonstrated a significant reduction of secondary caries formation [23].

In this study, a higher fluoride uptake was observed in the laser-SDF treated group compared with the group treated by SDF alone, especially after remineralisation. This suggests that remineralisation plays an important role in the fluoride uptake process. Previous studies have shown that fluoride uptake increases after laser treatment on both enamel and root surfaces [9,10]. One study that investigated the potential mechanism for the laser-fluoride effect on enamel found that low-energy Er-YAG laser irradiation coupled with fluoride treatment could inhibit enamel demineralisation through increased fluoride deposition on the surface and formation of fluoridated hydroxyapatite [11]. The fluoride weight percentage increased significantly after remineralisation, suggesting that fluoride played a more significant role in the remineralisation process than in the demineralisation process. An environment with a neutral pH and high concentration of Ca and P may favour fluoride precipitation. This effect also influences micro-mechanical changes, prompting a major increase in micro-hardness and elastic moduli after SDF application.

Higher Ca and P weight percentages were detected in the laser-treated groups in this study (Table 1), and no significant Ca/P differences were detected between any of the treatment groups. Previous studies have revealed similar results after laser treatment [24,25] and others have claimed that dentine apatite crystals melted and recrystallised into larger apatite crystals containing less carbonate during laser irradiation [24,26]. The SDF can react with hydroxyapatite and form fluoroapatite and insoluble silver phosphate, which may contribute to Ca/P ratio variations. Moreover, amorphous calcium phosphate might have been produced. The Ca/P ratio of amorphous calcium phosphate is variable and could contribute to the non-significance of the measured Ca/P ratio [27,4].

Nano-indentation has been used in measuring the mechanical properties of dental hard tissues in recent years because of the substantial improvement in indentation equipment and the need to measure on small scales [28-30]. It is now possible to monitor both the load and displacement of an indenter during indentation experiments in the respective micro-Newton and nanometre ranges with high precision and accuracy. Using these instrumented indentation techniques, the hardness and elastic modulus may be obtained from the displacement and the initial slope of the load-displacement curves [31]. Hardness is an engineering property and a measure of how resistant solid matter is to various kinds of permanent shape change when a force is applied

[32]. It is represented as the displacement of the load-displacement curve and therefore is an endpoint value. However, an elastic modulus is the mathematical description of an object or substance's tendency to be deformed elastically when a force is applied to it. It is defined as $\lambda = \frac{\text{stress}}{\text{strain}}$, where stress is the restoring force caused by the deformation divided by the area to which the force is applied, and strain is the ratio of the change caused by the stress to the original state of the object. Dentine is a non-Hookean material. The elastic modulus in the current study was obtained and calculated from the slope of the load-displacement curve [32]. Thus, the elastic modulus represents the process of loading and is an intrinsic material property. Smooth curves after remineralisation represent an improvement of the dentine surface's intrinsic property after remineralisation.

SDF inhibits biofilm growth [4], and the insoluble calcium fluoride may also precipitate as insoluble calcium fluoride, which can react with hydroxyapatite in dentine [33]. This could account for the higher micro-hardness value in group S compared with groups L and W in this study. Second, a relatively higher elastic modulus was found in group L than in groups S and W. Laser irradiation might have led to increased mechanical properties of the dentine due to water and organic component vaporisation [24], resulting in an increased surface micro-hardness after SDF application. In addition, laser improved the elastic modulus of the dentine, which was demonstrated in the displacement and slopes of the load-displacement curves. These could explain the better resistance to caries development of dentine treated with SDF application followed by Er:YAG laser irradiation.

Conclusions

In this study, SDF application followed by subablative low-energy Er:YAG laser irradiation on dentine rendered the dentine surfaces more resistant to caries development both chemically and mechanically.

Acknowledgement

This study was supported by the HKU Seed Funding Programme for Basic Research (Grant No. HKU#201111159079).

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Figure 1 Flowchart of experiment [In Fig. 1, 'remineralization' should be 'remineralisation'.]

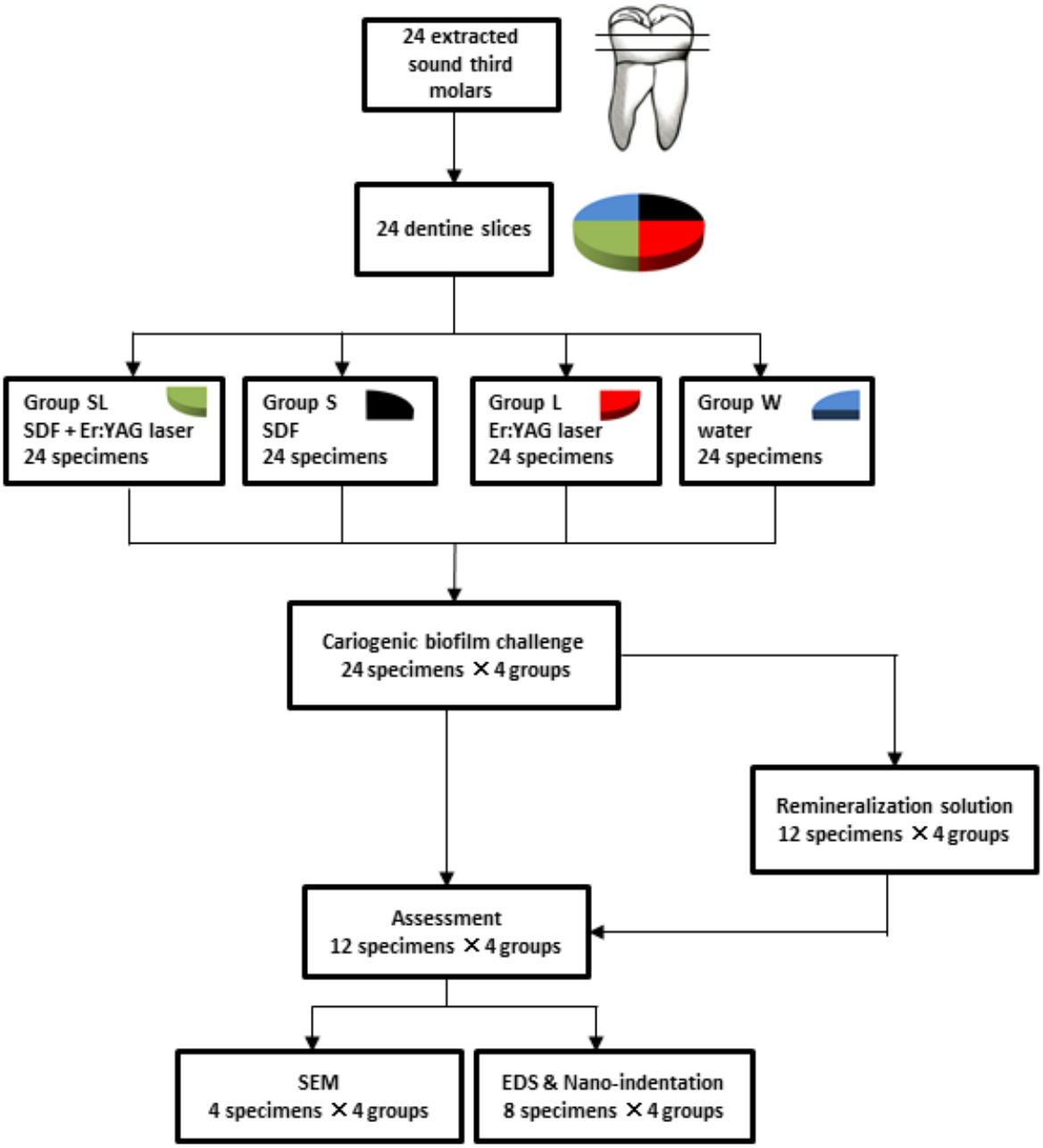

Figure 2 SEM images of dentine surface after cariogenic biofilm challenge and after remineralisation according to treatment group (magnification: ×1,500) [In Fig. 2, 'remineralization' should be 'remineralisation'.]

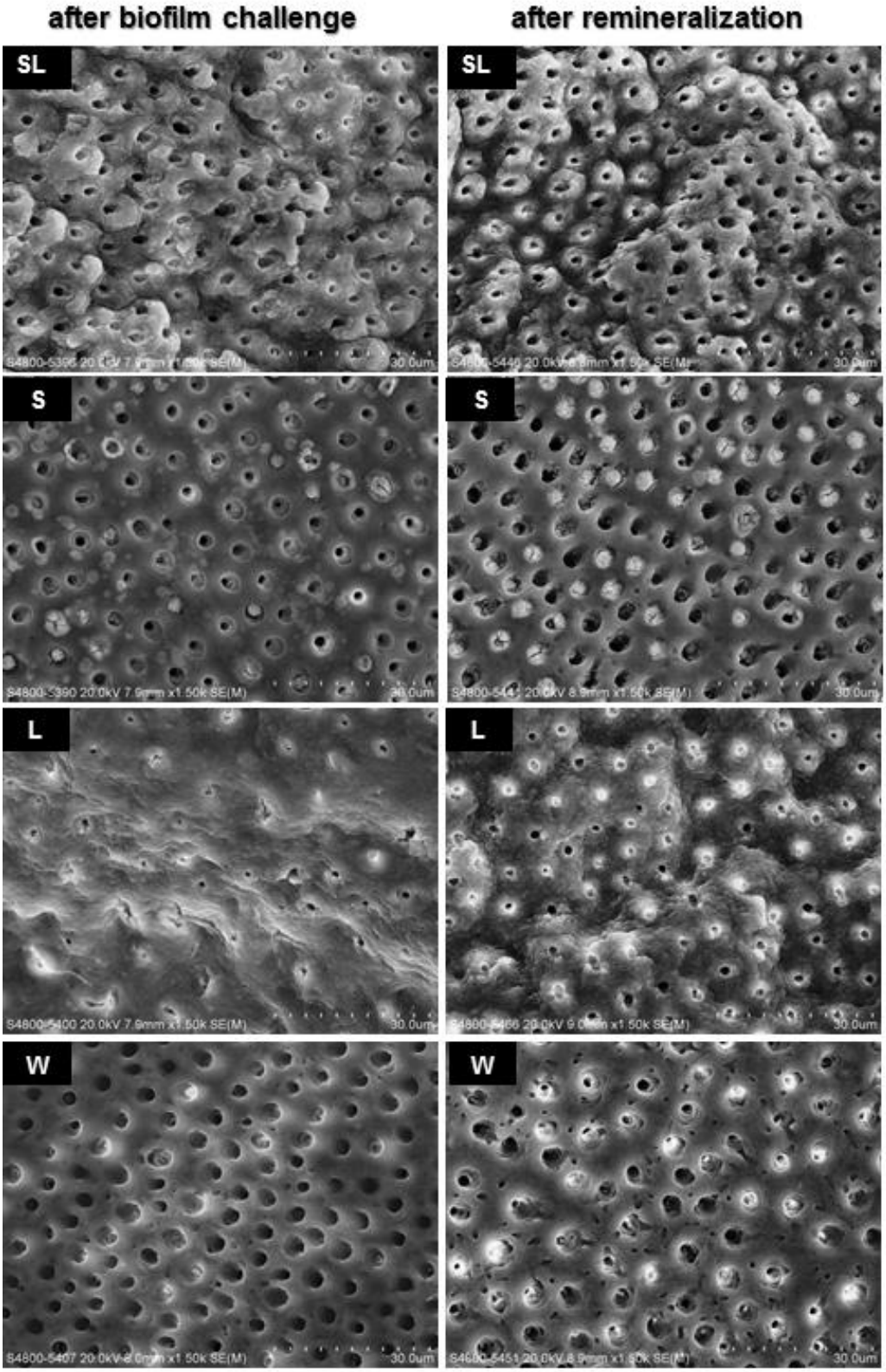
after biofilm challenge
after remineralization
SL
SL
S
S
L
L
W
W

Figure 3 Load-displacement curves of nano-indentation test on dentine according to treatment group (maximum loading force: 100 mN): A) after biofilm challenge, B) after remineralisation. [In Fig. 3b, 'remineralization' should be 'remineralisation'.]

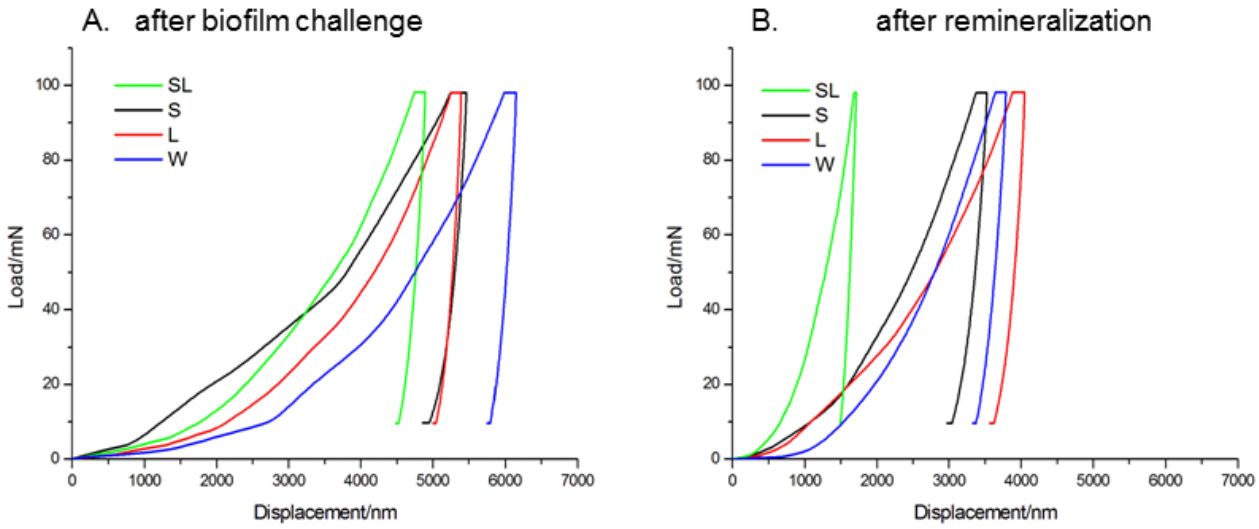

Table 1 Weight percentages of calcium, phosphorus, silver, fluoride and the Ca/P ratio (mean±SD) of dentine according to treatment group (n=8)

	Group				p value	LSD
	SL	S	L	W		
After cariogenic biofilm challenge						
Ag	5.78±4.61	6.21±5.10	0	0	<0.01	S,SL>L,W
F	2.45±0.71	0.95±0.22	0.14±0.60	0.09±0.9	<0.01	SL>S>L,W
Ca	24.9±3.52	22.4±3.46	24.0±6.09	20.2±3.09	<0.01	L,SL>W
P	13.1±1.21	13.4±2.25	14.1±1.66	10.8±2.15	<0.01	S,L,SL>W
Ca/P	1.90±0.19	1.71±0.30	1.70±0.36	1.80±0.30	<0.05	SL>S,L
After remineralisation						
Ag	6.72±5.97	5.99±6.24	0	0	<0.01	S,SL>L,W
F	3.93±0.91	3.10±0.61	0.17±0.09	0.32±0.07	<0.01	SL>S>L,W
Ca	26.1±4.50	23.0±4.38	23.8±3.12	21.7±7.54	<0.01	SL>W
P	13.1±2.11	11.7±3.11	13.9±1.94	10.8±3.61	<0.01	L>S>W SL>W
Ca/P	2.03±0.41	2.03±0.29	1.75±0.39	2.10±0.99	0.120	N/A

Table 2 Micro-hardness and elastic moduli (mean±SD) of dentine according to treatment group (n=8)

	Group				p value	LSD
	SL	S	L	W		
Micro-hardness (GPa)						
After biofilm challenge	0.20±0.03	0.17±0.02	0.13±0.01	0.11±0.01	<0.001	SL>S>L,W
After remineralisation	1.84±0.22	0.49±0.13	0.41±0.11	0.30±0.06	<0.001	SL>S>L,W
Elastic moduli (GPa)						
After biofilm challenge	16.2±1.3	13.2±1.0	16.0±0.9	12.7±1.2	<0.001	L,SL>S,W
After remineralisation	75.1±7.2	20.0±1.3	24.3±5.2	20.2±2.8	<0.001	SL>S,L,W