Quantitative Assessment Of Dentine Mineralisation And Tubule Occlusion By Novamin® And Stannous Fluoride Using Serial Block Face Scanning Electron Microscopy

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Abstract

Dentine hypersensitivity (DH) is one of the most common dental conditions affecting most adults during their lifetime. Tubule occlusion is a widely accepted method for treating DH. Current in-vitro techniques such as focused ion beam, scanning electron microscopy (SEM), or hydraulic conductance that are used to determine tubule occlusion do not provide the depth of occlusion, are time-consuming, expensive and the volume of dentine tested is limited. The presented study aimed to assess the ability of Serial Block-Face SEM (SBF-SEM) to section dentine, to quantify the number of occluded tubules including the depth of penetration by NovaMin® and Stannous fluoride (SnF_2) and to compare mineral density between the control and treated dentine. Results demonstrated that NovaMin® provided a better occlusion with 100% of the tubules blocked at the surface compared to 83% for SnF_2. The greyscale value (230.42) was significantly higher ($p \leq 0.05$) after treatment with NovaMin® compared to SnF_2 (222.06) and the control (196.37), indicating increased mineral density and dentine mineralisation. SBF-SEM has the potential to be used for large volume analysis of bone-like materials at high resolution with minimal sample preparation over a short period. It can be significantly useful in the development and research of new biomaterials.

Key Words: Novamin®, Stannous fluoride, Tubule occlusion, mineralisation, Serial block-face SEM

1. Introduction

Teeth can become sensitive for several reasons such as cracks, dental decay, and deep fillings. However, two of the main causes of dentine hypersensitivity (DH) are gum recession and thinning of the enamel due to over brushing or acid erosion, exposing dentine tubules. Tubules run through the entire structure of the dentine to the pulp and are filled with dentinal fluid. Stimuli such as pressure, temperature, and osmosis can alter the fluid flow within the tubules, distorting the nerve in the pulp. This initiates a neurological response resulting in a sharp pain experienced by individuals [1]. In the early stages, it is possible to manage and reduce sensitivity symptoms by using dentifrices designed to occlude these tubules by either depositing a layer over the exposed surface or by plugging materials down the tubules [2]. Stannous fluoride (SnF_2) [3–5] and NovaMin® [6–8] are two of the active ingredients used in toothpastes to occlude tubules and treat sensitivity symptoms. NovaMin® releases calcium and phosphate ions when it comes in contact with saliva, these ions get deposited onto the dentine surface and form a layer that is chemically and structurally similar to hydroxyapatite [9]. SnF_2 rapidly hydrolyses in the presence of saliva, fluoride ions are released and get incorporated into the hydroxyapatite structure forming a fluroapatite [10]. The stannous ions are then oxidised from Sn (II) to Sn (IV) and form insoluble oxides which plug the tubules [5]. He et al. [4] demonstrated that SnF_2 dentifrice provided an immediate and significant sensitivity relief in a clinical study compared to the sodium monofluorophosphate control. An in-vitro study by Takamizawa et al. [5] showed significant tubule occlusion after treatment with SnF_2. Earl et al. [11] used a focused ion beam (FIB) and scanning electron microscopy (SEM) to investigate the depth of tubules occlusion after treatment with NovaMin®. The results showed that dentine tubules were occluded to approximately 1μm (the depth of FIB cut). It is extremely time-consuming to mill a large volume of material to high depths by FIB-SEM. Earls et al. [12] used FIB-SEM to study the structure of the dentine where only 15 tubules were managed over 20 hours. SEM, on the other hand, does not provide information about the depth of occlusion. Serial block-face SEM (SBF-SEM) is a 3D imaging technique that uses a diamond knife in a microtome sitting within the chamber of an SEM to cut nanometre sized slices from the surface of a sample (block face). An image is initially taken from the block face, the sample is then cut at a given depth by the microtome, exposing a new block face. This process continues automatically and resulting images are compiled to provide a 3D reconstruction of the sample [13]. The present study aims to determine the level of tubule occlusion by NovaMin® and SnF_2, both by determining the number of occluded tubules per unit volume but also the depth of penetration of the active ingredients using SBF-SEM. It also aims to use the backscattered images from the SBF-SEM to investigate the mineralisation potential of the two ingredients using the greyscale values of the images which are proportional to the mineral density [14].

2 Materials and methods

2.1 Dentin discs and toothpaste treatment

Bovine dentine discs (n=6) with a thickness of 1.00 ± 0.3 mm were supplied by Modus Laboratories Ltd (Reading, UK). Each disc was polished using 3µm and 1µm diamond suspensions to get a smooth surface finish, etched with 1% citric acid for 30 seconds to remove the smear layer and washed with deionised water for 1 minute. The discs were then randomly divided into two equal treatment groups (n=3). Discs were halved with one half treated with either Sensodyne® Repair and Protect (NovaMin®) or Rapid Relief (SnF_2) toothpastes (table 1) and one half as control. 2.0 ± 0.1mg of respective toothpaste was weighed onto a medium manual toothbrush (Colgate) and brushed on to the dentine discs for two minutes, twice per day for 7 days. 0.50ml of artificial saliva (AS) with a pH of 6.5 was pipetted on to the discs to stimulate the reaction of active ingredients in conditions that were more representative of the oral environment. The control discs were only brushed with AS. All specimens were kept in AS between brushings. The AS contained carboxymethylcellulose (5g), glycerol (50g), DI (1L) sodium phosphate dibasic dodecahydrate (0.58g), urea (1g), NaCl(0.4g), KCl(0.4g) and CaCl2 (0.6g) [15].

Table 1: Toothpastes and their active ingredient used in this study

Toothpaste	Active ingredient	Other ingredients	Manufacturer
Sensodyne® Repair and Protect	NovaMin® (calcium sodium phosphosilicate) 5% w/w	glycerin, PEG-8, hydrated silica, cocamidopropyl betaine, sodium methyl cocoyl taurate, aroma, titanium dioxide, carbomer, sodium saccharin, sodium fluoride (1450ppm)	GlaxoSmithKline
Sensodyne® Rapid Relief	Stannous fluoride (SnF_2), 0.454% w/w	glycerin, PEG-8, hydrated silica, pentasodium triphosphate, aroma, sodium lauryl sulfate, titanium dioxide, carbomer, stannous fluoride, cocamidopropyl betaine, sodium saccharin, sodium fluoride, limonene., sodium fluoride 0.072% w/w (1450ppm fluoride).	GlaxoSmithKline

2.2 SBF-SEM imaging and processing

After the 7 days of brushing treatment, dentine discs were air-dried at room temperature (21–23 °C, 24hr), and 0.5 by 0.5 mm blocks were cut out from the discs. The blocks were infiltrated with resin and polymerised at 60 °C overnight (Agar low viscosity (ALV) resin, Agar Scientific, Stansted, UK). The excess resin was trimmed away and the block glued on to aluminium pin using conductive glue before sputter coating with gold/palladium to increase the conductivity of the block. Blocks were imaged using a Gatan 3View (Gatan, Abingdon, UK) inside a FEI Quanta 250 FEGSEM (Thermo Fisher, Eindhoven, the Netherlands) at 2.5 kV accelerating voltage, and with a vacuum level of 30 Pa. Stacks of 6000 x 6000 pixel images were collected at a pixel size of 19 nm resulting in a 114 x 114 µm field of view. 600 slices were cut by an ultrasonic diamond knife with a thickness of 60nm during data acquisition resulting in an imaging depth of approximately 36µm. After each slice, the dentine surface was imaged with a backscatter (BS) electron detector. Once the images were collected they were processed and analysed using ImageJ software. All tubules (open or occluded) were identified using the segmentation function, their position was subsequently recorded throughout the stack and it was used to align the data set and straighten each tubule. Once the tubules were identified and straightened, the level of the blockage was evaluated by scanning through the core of each one and recording the greyscale values (Figure 1). As demonstrated in the example given in figure 1 there is a big drop in greyscale value to below 40 at around 7µm when the line moves past the occluded portion of the tubule. Therefore this value was chosen as the benchmark to separate filled vs unfilled tubules.

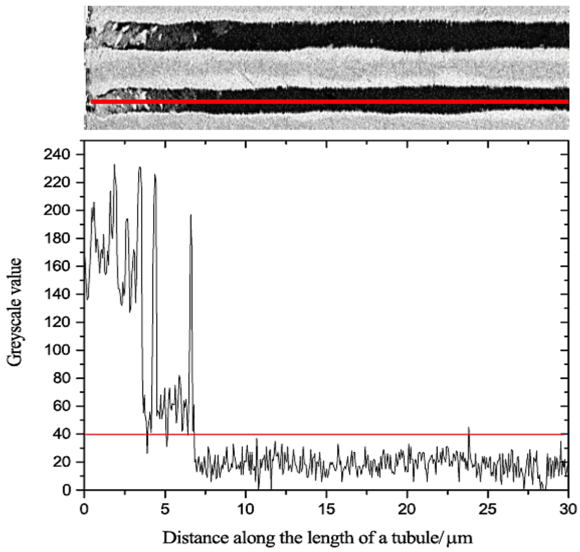

Figure 1: XZ and YZ planes for a tubule and grey values taken by scanning the core of tubule

2.3 Remineralisation potential and diameter of tubule opening

To compare the mineral density of the dentine samples the greyscale values of the BSE images were taken from the block face by ImageJ software. The greyscale values from 100 random areas around the tubules were recorded at every 3µm from the surface to the depth of 30µm. After each tubule was identified, a stack of all images for that tubule was made and a thickness graphic mask was applied to generate a heat map. The diameter of these heat objects was then measured throughout the stack which represented the open section of the tubule. Areas that had a grayscale value of more than 40 (occluded) were thresholded to appear black and excluded from the measurement. The outputted values included the highest, lowest, mean and standard deviation diameter throughout the entire stack.

2.4 Statistics

The data was initially analysed using a one-way analysis of variance (ANOVA) to determine significance. This was followed by a t-test (assuming equal variance) to identify any significant differences between the means of the groups. ($P \leq 0.05$) was considered to be significant.

3. Results

3.1 Tubule occlusion and mineralisation

The SEM images (Figure 2) taken before slicing showed that both toothpastes were successful in occluding tubules compared to the control samples. NovaMin® formed a layer over the tubules (figure 2b) whereas there was no layer formed by stannous fluoride (figure 2c). Cross-sections of the tubules (figure 2, right hand images) showed that the tubules within the control group were empty. Tubules treated with NovaMin® and SnF_2 contain occluding material throughout the length of the tubules at various depths. The level of tubule occlusion reduced as the distance from the surface increased and at 3µm, the percentage of tubules with occlusion was significantly ($P \leq 0.05$) reduced for both treatment groups. Although NovaMin® had better occlusion at the surface SnF_2, occluded more tubules between 3 and 24µm from the surface. However, between 24-30µm NovaMin® had a superior occlusion rate although the difference was non-significant ($P \geq 0.05$) (figure 3L). Greyscale values around the tubules (figure 3R) were significantly higher ($p \leq 0.05$) for NovaMin® treated samples at the surface and 30µm below the surface (230.42 and 213.55 respectively) compared to SnF_2 (222.06 and 192.76) and the control (196.37, 192.35).

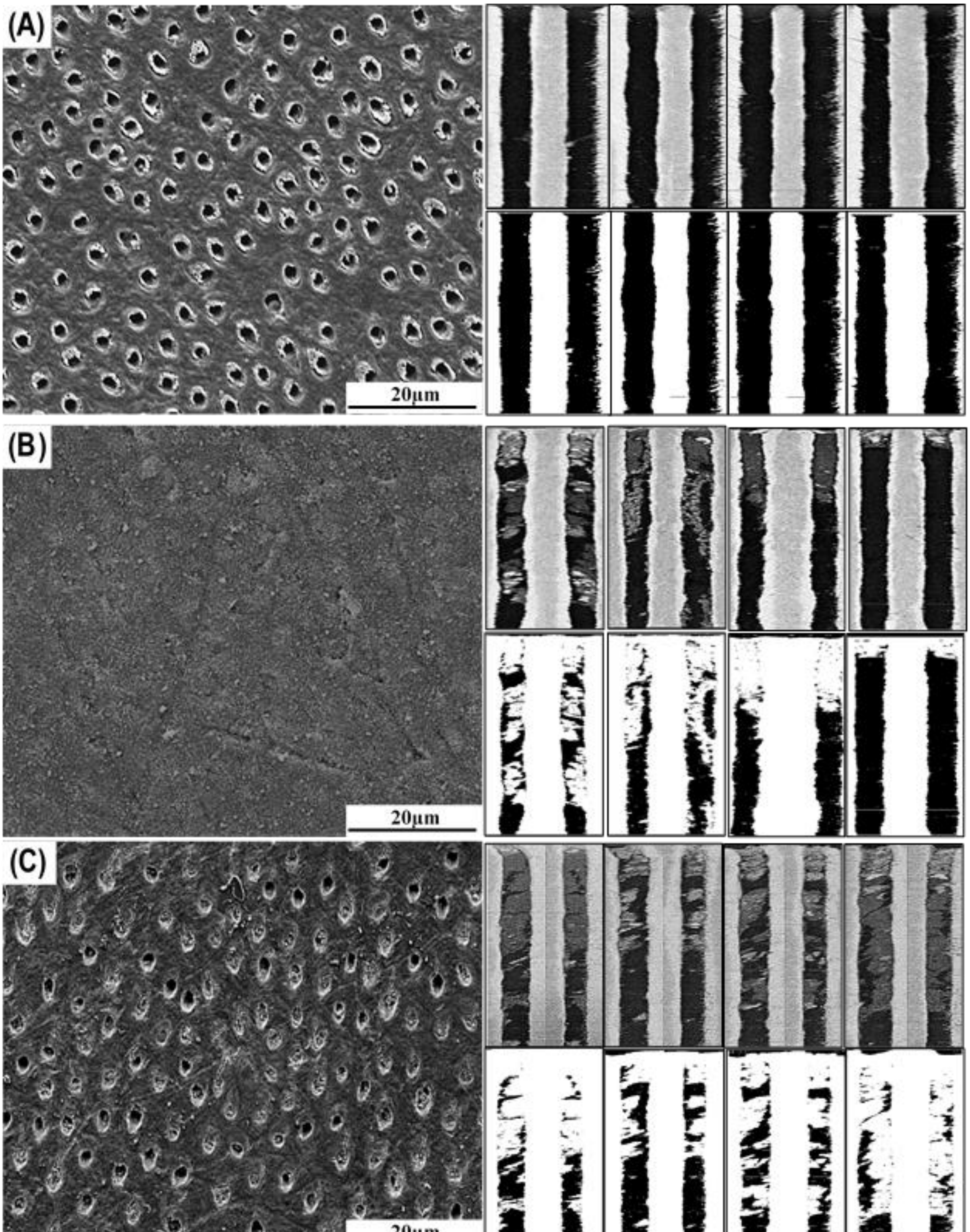

Figure 2: SEM images of control (a), NovaMin® (b), and SnF_2 (c) treated dentine surface, images to the right are the cross-section of random tubules taken from the respective groups. Top row: each pair shows XZ (left) and YZ (right) planes for a tubule. Bottom row: tubule was 3D-flood-filled from the bottom

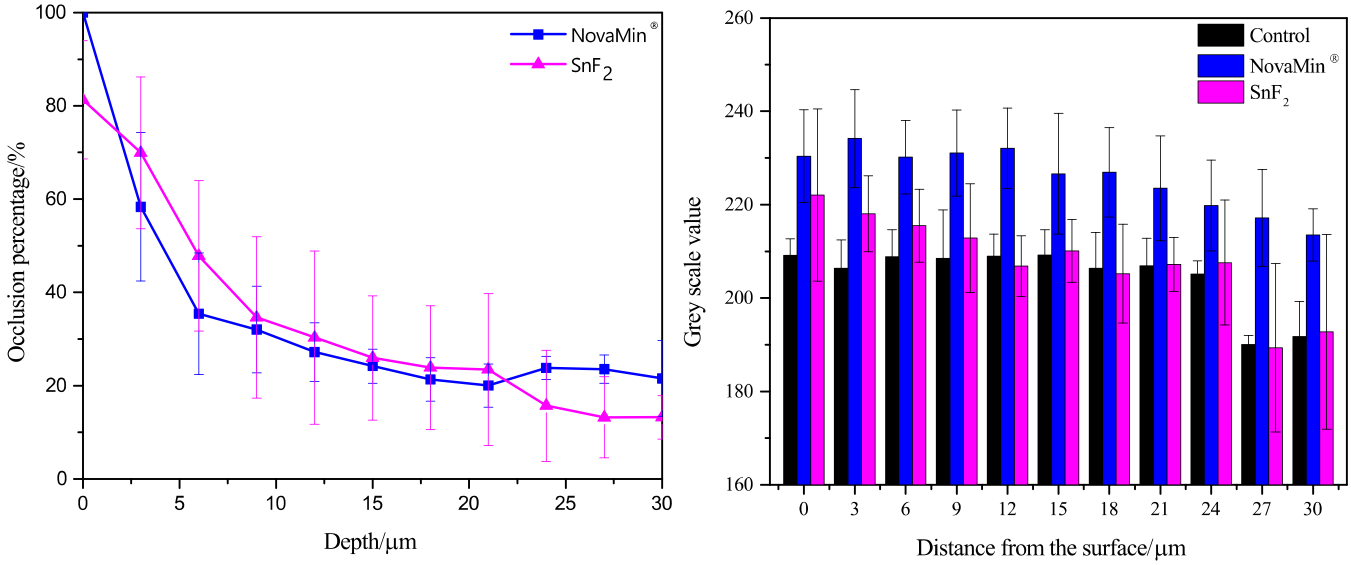

Figure 3: Percentage of blocked tubules (L), greyscale values of control and treated dentine (R)

3.2 Tubule Diameter

The diameter of tubule opening (figure 4L) was significantly ($p \leq 0.05$) reduced from 0.79±0.05µm to 0.66±0.21 µm after treatment with NovaMin®. No significant ($p \geq 0.05$) change was seen after SnF_2 treatment. Figure 4R was taken from a NovaMin® treated sample after it had been through a cutting and imaging process (30 µm was removed from the surface). The growth of material around the tubule is evident (red arrow) and can explain the reduction in the diameter of tubule opening.

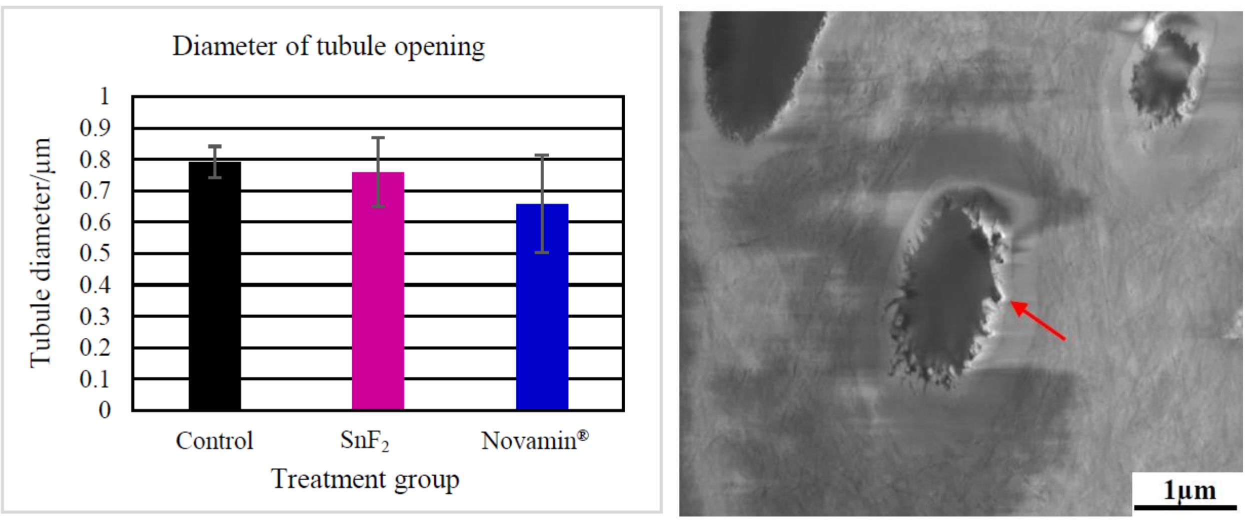

Figure 4: Diameter of the tubule opening (L) and SEM image taken from a NovaMin® treated dentine disc after 30µm was removed from the surface (R)

4. Discussion

For prevention and treatment of dentine hypersensitivity (DH) the permeability of the tissue needs to be reduced to inhibit stimuli from altering the fluid flow and stimulating the nerve endings inside of the tooth. This can be achieved by occlusion of the exposed dentine tubules. In the present in-vitro study, serial block-face scanning electron microscopy (SBF-SEM) was used to compare dentine tubule occlusion by Sensodyne® Repair and Protect and Rapid Relief toothpaste containing NovaMin® and stannous fluoride (SnF_2) respectively as active ingredients to treat DH. The backscattered electron (BSE) imaging also made it possible to compare the mineral density of the dentine tissues between the control and the treated samples. SBF-SEM imaged approximately 100 tubules per sample with a 19 x 19 x 60 nm voxels size over approximately 12 hours. This resulted in an imaging volume of approximately 4.4355 x 105μm^3. The SEM data showed that both materials were capable of occluding a significant number of tubules, while in the control group they were still open. However, the occlusion percentage decreased with increasing depth away from the surface. NovaMin® produced a better level of occlusion both at the surface and at 30µm. It also resulted in a reduction in tubule opening by forming a layer down the walls of the tubules. The greyscale values around the tubules were higher for NovaMin® treated samples at each given depth compared to the control and SnF_2. These results are similar to studies in literature, Parkinson et al.[16] used SEM to investigate the occlusion and mineralization of NovaMin® and SnF_2. They reported that NovaMin® containing toothpaste had a significantly higher level of dentin tubule occlusion and mineralisation to compare to SnF_2 after being brushed for two minutes twice per day over four days. An in-vitro SEM study by Kulal et al. [17] reported 98.1% dentine tubule occlusion following a 7 day (two minutes per day) treatment with a NovaMin® containing toothpaste. whereas another in-vitro SEM study reported 92.73% occlusion after brushing with NovaMin® containing toothpaste for 6 min twice a day for 7 days [18]. A recent in-vitro SEM study by Hines et al. [19] reported that SnF_2 containing toothpaste occluded 82% of tubules. A clinical study comparing NovaMin® and SnF_2 for treating DH also reported that NovaMin® reduced DH significantly more than SnF_2 after 12 weeks [20]. The findings in this study encourage the use of NovaMin® containing toothpaste over a toothpaste with SnF_2 as an active ingredient due to a better occlusion and mineralisation potential of NovaMin®. Also, SBF-SEM enabled the study of a much larger volume of dentine over a short period with high resolution. To analyse a similar number of tubules at this resolution with other techniques such as focused ion beam (FIB) SEM would have required significantly more sample preparation which would be time-consuming, costly and the depth would be limited to the FIB slice.

5. Conclusion

NovaMin® and SnF_2 containing dentifrice have the ability to occlude dentine tubules, with NovaMin® able to occlude 100% of the tubules due to penetration of material into the tubules and the formation of the surface layer. The NovaMin® and SnF_2 both increased the dentine mineralization level as quantified by greyscale values from the backscattered images. The increased level of tubule occlusion and dentine mineralisation by the NovaMin® containing dentifrice may provide better relief and protection from dental hypersensitivity.

SBF-SEM is a high resolution imaging technique which can provide a new quantitative method of assessing dentine tubule occlusion depth and percentage of occluded tubules, while also allowing for the assessment of mineralisation changes within the treated tissues.

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Conflict of interest

The authors declare no conflict of interest

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Author contributions

Author 1: Contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript

Author 2: Contributed to design, data acquisition and critically revised the manuscript

Author 3: Contributed to conception, design, and critically revised the manuscript

Author 4: Contributed to conception, design, analysis and interpretation, critically revised the manuscript

All authors gave their final approval and agree to be accountable for all aspects of the work.

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