Quantitative Assessment of Desensitizing Agents in Occluding Dentine Tubules using Image Analysis.

Ip T[1], Gillam DG[1*], Barber PM[2], Bulman JS[3], Mordan NJ[3].

[1]Department of Periodontology, Eastman Dental Institute for Oral HealthCare Sciences, 256 Gray's Inn Road, University of London, London WC1X 8LD, UK.

[2] Electron Microscopy Unit, Eastman Dental Institute for Oral HealthCare Sciences, 256 Gray's Inn Road, University of London, London WC1X 8LD, UK.

[3]Department of Dental Public Health, Eastman Dental Institute for Oral HealthCare Sciences, 256 Gray's Inn Road, University of London, London WC1X 8LD, UK.

*Corresponding author: Dr. David Gillam, Oral Bioengineering, Institute of Dentistry, Barts and the London School of Medicine and Dentistry QMUL, London, E12AD; UK.

Copyright: © 2020 Dr David Gillam

Abstract

Previous in vitro studies assessing the tubule occluding properties of various desensitizing agents in the dentine disc model appear to provide only qualitative data. The aim of this study was to establish a reliable and reproducible system to evaluate the in vitro effectiveness of three desensitizing agents. Six selected fields from SEM negatives (magnification x1000; working distance 10mm) of test and control dentine disc specimens treated with the desensitizing agents (Butler Protect, Colgate FluoriGard (Gel-Kam), Macleans Sensitive) were evaluated. Fields were assessed using a Quantimet 520 Image analysis system (Leica UK) and the data recorded included patent tubule area, mean tubule diameter, mean patent/field area and number of tubules per unit area. Comparison of test and control specimens indicated that differences in the number of tubules, patent area, width of tubules and percentage of patent areas can be assessed quantitatively Furthermore, this methodology also demonstrated differences between the tubule occluding properties of the selected desensitizing agents.

Keywords: Dentine Hypersensitivity, Desensitizing Agents, Image Analysis, Tubule Occlusion

Introduction

Dentine hypersensitivity (DH) may be defined as pain arising from exposed dentine, typically in response to chemical, thermal or osmotic stimuli, that cannot be explained as arising from any other form of dental defect or pathology [1-2]. Prevalence rates of this painful experience in various self-reporting surveys appear to involve 8-35% of populations in different countries [3]. Methods of treating DH may be classified either according to their mode of action [4], their physical or chemical attributions [5] or more generally as in-office or Over-The-Counter (OTC) products. Currently, the most accepted mechanism of intradental nerve activity associated with DH appears to be hydrodynamic in nature [6]. The concept of tubule occlusion as a method of dentine desensitization therefore, is a logical conclusion from the hydrodynamic theory [7]. Previous in vitro studies have assessed the tubular occluding effects of various desensitizing agents by scanning electron microscopy (SEM). However, with few exceptions, these have only provided qualitative results. There have been several previous studies [8-9] claiming quantification by assessing the number of open tubules on micrographs, which have provided only semi-quantitative data. The aim of this study was to develop a reproducible quantitative model using SEM and image analysis to evaluate the in vitro effectiveness of selected desensitizing agents.

Material and Methods

Unerupted caries-free surgically extracted human third molars were collected from the Maxillo-Facial Surgery Department, Eastman Dental Hospital, London UK and, fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer solution (pH7.4) at 4°C for one week. After cleaning the teeth by removing any organic material, teeth were sectioned mesio-distally into 1mm discs using a diamond-edged cutting machine (Test-Bourne model 660, Cambridge, UK)(Figure: 1) and stored in cacodylate buffer solution at 4°C for one to two weeks.
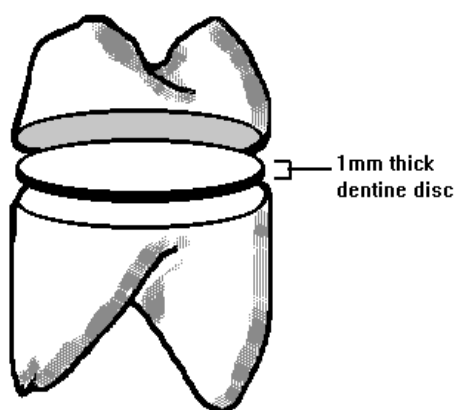

Figure1: Preparation of a dentine disc from a human third molar

The discs were ultrasonicated in distilled water for 30 seconds, etched in 6% citric acid on a rotator for two minutes and rinsed in distilled water. Marks were made with indelible ink on either side of the discs to identify control and test sides. The discs were then fractured in half using a chisel, to provide control and experimental test sections. The control side was rinsed with distilled water for 10 seconds and allowed to dry in a desiccator for one day and the test side was soaked in saliva supernatant and desensitizing agents applied. The discs were either brushed with test agent for two mins or an aqueous solution was applied as per manufacturers' instructions. The discs were rinsed in distilled water for 10 sec. to remove excess slurry or aqueous solution and allowed to dry in a desiccator for one day. The specimens were mounted onto aluminum stubs, coated with gold/palladium in a Polaron E5200 sputter coater (BioRad U.K.) and viewed with a Cambridge 90B Scanning Electron microscope (Cambridge U.K.). As described in Mordan et al. [10] micrographs were only taken from the centre of the disc halves to ensure similarity of tubular size and orientation in order to obtain a good control comparison. Four micrographs from each side of the specimen were taken (Figure. 2) at the same magnification (about 1,000X) and viewing distance (10mm) for quantitative analysis using a Quatimet 520 image analysis system (Leica, U.K.).
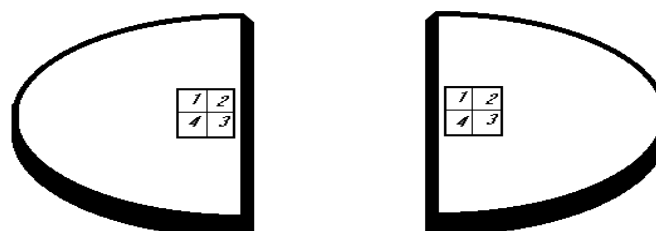
1. Dentine disc split into halves: control and test

2. Specimens prepared for SEM viewing
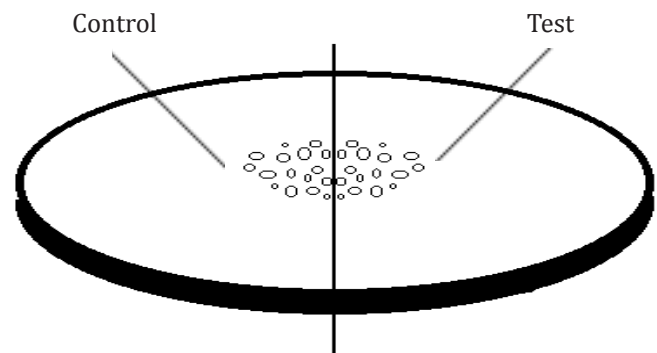
3. SEM: Disc centre portion location
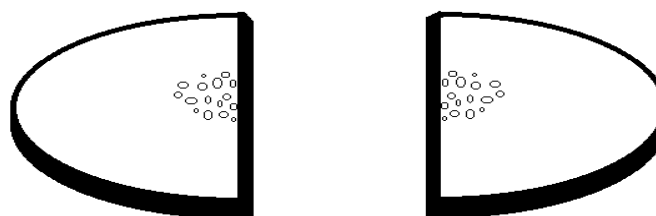
4. Micrographs taken in 4 areas

5. Each micrograph divided into 6 fields for image analysis

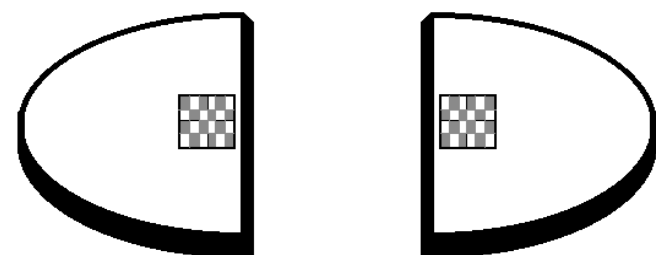**Figure 2:** Flow diagram illustrating how fields of dentine tubules were obtained for image analysis.

Quantitative study
Image analysis system

Image analysis utilized a computer-based processing system which involved the extraction of quantitative information from binary images. Negatives were placed on a light box (Model: QUP/A4SL 34.5 x 27.5 x 9.5cm) on a base of the copy

stand and viewed using a high resolution monochrome Vidicon-Chalnicon camera coupled via a C-mount connector to a Tamron 35-80 mm camera lens with 1X, 3X and 4X attachment lens. The video was positioned a constant 520mn above the copy stand. A grid of six fields was placed onto the negative in order to measure the same areas when they were to be repeated. The camera transmitted an image of the micrograph, through the image processor (Quatimet 520 image analysis system Leica, U.K.) to the image display monitor. A Houston Instrument Hipad™, digitablet, connected to the image analysis processor, was used to precisely identify areas to be measured. The M420 Olivetti personal computer, attached to the image analysis processor, stored all feature data and image files during each analysis session. The computer was connected to an Epson FX-1000 printer which was used to provide a running hard copy of all data as collected (see Figure: 3).
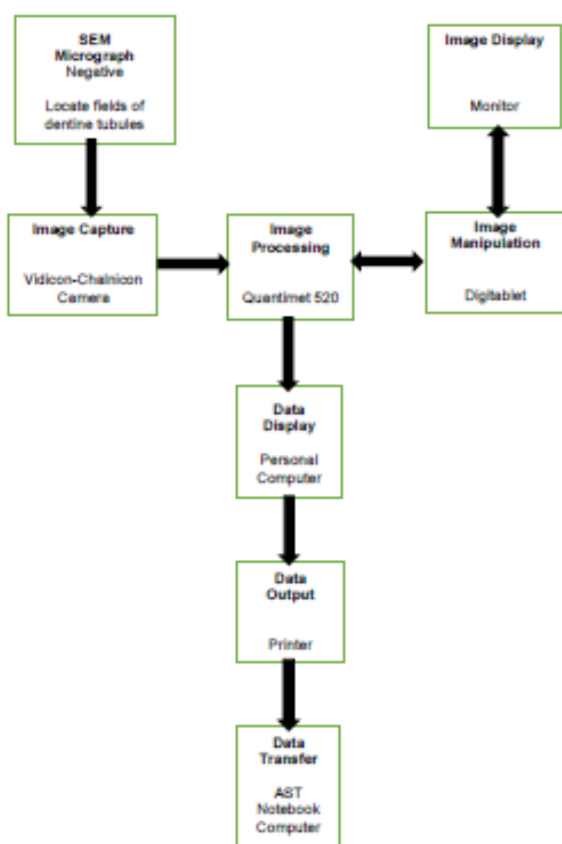

Figure 3 Flow diagram of the image analysis system used in the study

Figure 3: Flow diagram of the image analysis system used in the study

Image setup, calibration and use of the Image analyzer (Q520)
Capturing of Image
The following methodology was used:
- Image set-up of Gain = 8 and offset = 0
- Image frame and frame adjustment to ensure that a sufficient area was measured

- Calibration set-up of 20μm bar was used as a standard as the start of each session and for each new set of micrographs (0.062-0.668μm per pixel)
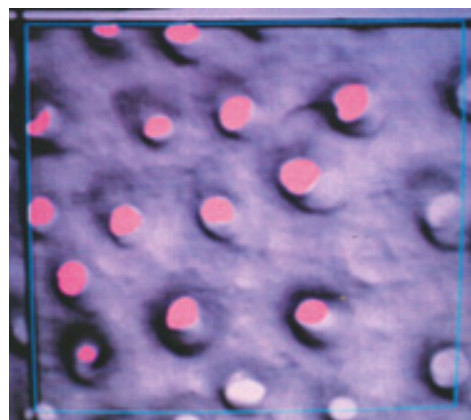- Detect image
- Edit image using the digitablet (Figure: 4)

Figure 4: Identifying the patent areas of dentine tubules in the measuring frame.

- Measure all detectable features/tubules (Figure: 5)

Figure 5: Display of measurements of the features.

- Each feature/tubule was numbered and data collected in μm and included
  1) Patent tubule area
  2) tubular diameter
  3) frame area and
  4) total detected area

In the pilot study only three different fields were chosen at random.

When it was observed that the two runs of measurements on the test side of the specimen of the Sensodyne Sealant were significantly different, a grid of six fields was placed on the micrograph in order to:
a) cover evenly the whole area to be measured and
b) measure the same area in the two runs.

All measurements were downloaded to the printer and then transferred to the notebook computer for further calculations and storage of data. The following data were collected, calculated and analyzed:

- Patent tubule area
- Mean tubules diameter (width)
- Mean patent area/field area (fraction)
- Number of tubules per unit area (count)

Pilot Study

Different magnifications of two desensitizing agents (Sensodyne Sealant [Block Drug Inc, NJ, USA now GSK, UK] and Butler Protect [JO Butler, Chicago, USA now Sunstar Pharmaceuticals, Japan]) were compared: 500x, 1,000x, 2,000x and 4,000x and a magnification of 1,000x was found to provide reasonable numbers of tubules in a field to be counted and details of the tubular walls were clear for individual identification. Working distance was set to 10mm and a 20 μm bar was included for calibration. It was decided to measure the negatives placed on the illumination box rather than using micrographs. The 20 μm bar was calibrated to 1100 μm to reflect the actual length. Three fields chosen at random per negative were measured. Comparisons of the control and test sides and the repeated measurements of the same specimen were made.

Main study

Three different types of desensitizing agents, namely: Maclean Sensitive toothpaste (SmithKline Beecham, now GSK, UK), Colgate FluoriGard (Gel-Kam) (Colgate-Palmolive (UK) Limited) and Butler Protect (JO Butler, Chicago, USA, now Sunstar Pharmaceuticals, Japan]) were used in the main study. These formulations and products used in the present study however, may no longer be available. Based on the methodology developed in the pilot study, measurements of patent tubule areas were obtained. Calibration of the 20 μm bar was set to 20 microns to obtain a direct measurement. Four negatives were used for each control and test run.

Statistical analysis

All data were stored and analyzed using:

1. Microsoft Excel (Version 5.0 and 4.0 spreadsheet)
2. SPSS-Windows (Version 6.11)

Comparisons were made between data obtained from
1. Control and test specimen;
2. The two sets of measurement of the specimen.

Descriptive analysis included:
1. Arithmetic mean and Standard deviation (S.D.)
2. 95% Confidence Intervals (95% C.I.)

Minimum level of significance selected was 0.05 (95% Confidence Level).
Independent mean sampling t-tests were used.

Results

Two desensitizing agents; namely Butler Protect and Sensodyne Sealant were used in the pilot study and three desensitizing agents, namely: Butler Protect, Macleans Sensitive toothpaste and Colgate FluorideGard (Gel-Kam) were used in the main study. Both studies were repeated to test for reproducibility. Measurements of the number of tubules (no. tub), width of tubule lumen (width), tubule patent area (patency) and proportion of patent area against field area (fraction area) were obtained and compared.

The pilot study was undertaken to assess the reproducibility of the technique. Two test products were chosen for their different microscopic appearance after application to the dentine disc. The results from this pilot study indicated that where the surface effects were minimal that was good statistical reproducibility, however where there was good occlusion, the method was not reliable (Tables 1 & 2).

Table 1 Summary of results from the pilot study

	No. Tubules		Mean Area (μm²)		Mean Width (μm)		% Area	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Butler Protect	112	109	2.3+1.4	2.7+1.8	1.73+0.6	1.71+0.6	24.9	23.4
Control Test	106	101	1.1+0.8	1.1+0.9	1.04+0.2	0.97+0.3	10.3	9.0
Sensodyne Sealant	112	117	2.48+1.3	2.48+0.7	1.60+0.2	1.71+0.6	23.9	24.2
Control Test	6	11	2.20+0.3	0.87+0.2	1.60+0.3	1.68+0.6	1.10	0.80

Table 2   Comparison of mean areas obtained in the pilot study.

Butler:			
1st run	Control vs	Treated	=sig. diff. (p=0.02)
	vs	vs	
2nd run	Control vs	Treated	=sig. diff. (p=0.02)
	=sig. diff. (p=0.02)	=sig. diff. (p=0.02)	
Sensodyne Sealant:			
1st run	Control vs	Treated	=sig.diff. (p=0.02
	vs	vs	
2nd run	Control vs	Treated	=sig.diff. (p=0.01)
	=no sig. diff. (p=0.5)	=*sig. diff. (p=0.01)	

This was due to the fact that when there are few open tubules per negative, measuring fields at random meant that some fields having no open tubules whereas some had up to two or three open tubules, thus giving rise to large apparent inaccuracies.

Therefore, in the main study, a grid was placed over the negative in order that the same areas were analysed and compared. In this study, three products were examined and compared with their own control half-discs and with each other. The results indicated firstly that the technique was reproducible (Table 3) and secondly that all agents occluded when compared with their controls, even when this was not always evident at a qualitative level (Table 4 and Figure: 6).

Table 3   Summary of results from the main study

	No. Tubules		Mean Area (µm²)		Mean Width (µm)		% Area	
	1st	**2nd**	**1st**	**2nd**	**1st**	**2nd**	**1st**	**2nd**
Protect Control	440	452	5.35 +1.9	5.25 +1.9	2.71 +0.6	2.65 +0.6	7.9	8.2
Test	375	373	4.24 +1.7	4.07 +1.7	2.38 +0.5	2.35 +0.5	5.6	5.2
Maclean Control	443	443	5.77 +2.4	5.47 +2.4	2.54 +0.6	2.50+0.6	8.4	8.0
Test	370	376	4.43 +2.1	4.58 +2.2	2.44 +0.7	2.42+0.6	5.7	5.7
GelKam Control	382	391	3.84 +1.9	3.66 +1.8	1.97 +0.7	2.01 +0.5	6.6	5.7
Test	325	320	2.26 +1.1	2.36 +1.1	1.86 +0.5	1.91 +0.5	3.0	3.0

Table 4   Average density of dentine tubules of the control discs used in the study

	Protect	Sensitive	GelKam	Total	Average
a.Field area	1238	1257	1036	3531	1177
b.Total (a x24)	29712	30168	24885	84765	28255
c.No.of tubules	440	443	382	1265	421
d.Tub/1000 µm²	14.8	14.6	15.3	44.7	14.9
e.Tub/1 mm²	14808	14684	15350	44842	14947

Patent Tubular Areas
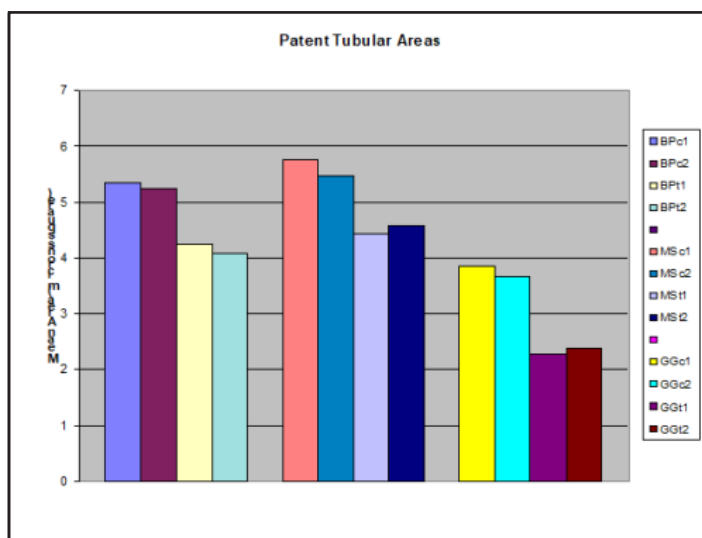
BP= Butler Protect MS=Macleans Sensitive GG=Colgate Fluo-riGard

c1= control 1st run
c2= control 2nd run
t1= test 1st run
t2= test 2nd run

Figure 6: Patent tubular areas of control and test specimens

Pilot study
Control vs test sides
      The results for both Butler Protect and Sensodyne Sealant showed consistent decreases in the number of open tubules, patent areas, width of tubules and percentage of patent areas against the field measured (Tables 1 & 2). Sensodyne Sealant showed that there were marked decreases in tubule numbers and % patency, but there was no apparent difference in mean areas or tubule width between the control and test specimens. This may relate to the tubule occluding characteristics of Sensodyne Sealant by which the agent is supposed to block the whole tubule rather than narrow the tubule lumen [11].

Reproducibility test
a) All parameters showed no differences when the first and second sets of measurements using Butler Protect for control and test sides were compared.
b) Similar results obtained for the two sets of measurements for Sensodyne Sealant on the control side.
c) There was a significant difference (p= 0.01) between the two sets of measurements for both Butler Protect and Sensodyne Sealant on the test side (Table 2).

      As a result of the lack of significance between the first two sets of measurements, another examination of the Sensodyne Sealant test side was undertaken. Since the sealant showed a remarkable occluding effect which could be visualized from the micrographs, leaving only a few unoccluded tubules in a field, a minor difference between the two examinations becoming significant, two modifications to the meth-

odology were therefore introduced for the main study:

1.   The number of fields was doubled to six
2.   A grid of six fields was created and placed over the micrograph to establish the area to be examined

Main study
The results may be summarized in the following manner (see also Tables 3-4, 5a-5c):

Table 5a: Density of dentinal tubules tested with Butler Protect

	Control		Test	
	1st Run	**2nd Run**	**1st Run**	**2nd Run**
a.Field area, μm^2	1238	1204	1172	1204
b.Total area, μm^2 (a.x 24 fields)	29712	28896	28128	28896
c.No.of tubules	440	452	375	373
d.Tubules /1000μm^2 (c/b x 1000)	14.8	15.6	13.3	12.9
e.Tubules/1 mm^2 (d. x1000)	14808	15642	13331	12908

Table 5b Density of dentinal tubules tested with Macleans Sensitive

	1st Run	2nd Run	1st Run	2nd Run
a.Field area, μm	1257	1240	1190	1248
b.Total area, μm^2 (a.x 24 fields)	30168	29760	28560	29952
c.No.of tubules	443	443	370	376
d.Tubules/1000μm^2 (c/b x 1000)	14.6	14.8	12.9	12.5
e.Tubules/1 mm^2 (d. x1000)	14684	14885	12955	12553

Table 5c : Density of dentinal tubules tested with Gel-Kam

	Control		Test	
	1st Run	2nd Run	1st Run	2nd Run
a.Field area, μm^2 b.Total area, μm^2 (a.x 24 fields)	1036 24885	1036 24885	1036 23885	1105 26527

07

Density of dentinal tubules
	The total number of tubules counted in the three control discs was 1265 over a total area of 84765 μm^2. The average number of tubules per 100 μm^2 was 14.9. This density corresponds to 14947 tubules per mm^2.

Area of patent tubules (patency)
	The average patency for the three control specimens was (5.35+5.77+3.84)/3= 4.98 μm^2, whereas the average for the test specimens was (4.24+4.43+2.26)/3= 3.64 μm^2. There was a consistent decrease in patency in all test specimens which can be interpreted as a result of the effectiveness of the desensitizing agents (Figure: 6).

Width of dentinal tubules
	The average width for all the control specimens was (2.71+2.54+3.66)/3= 2.97 μm whereas the average for all the test specimens was (2.38+2.44+1.86)/3= 2.22 μm. There was a consistent decrease in tubule lumen width after the application of desensitizing agents to the dentine discs.

Fraction of patent areas vs measuring fields
	The average fraction of the patent areas against the measuring fields in all control specimens was (7.9+8.4+6.6)/3= 7.6% whereas the average results in all test specimens was (5.6+5.7+3.0)/3= 4.7%.

Differences between control and test specimens
	All results showed consistent significant decreases in the test specimens for all the parameters measured, namely:
- number of tubules (no. tub)
- width of the tubule lumen (width)
- patent area of the tubules (patency) and
- proportion of patent area against field area (fraction area)

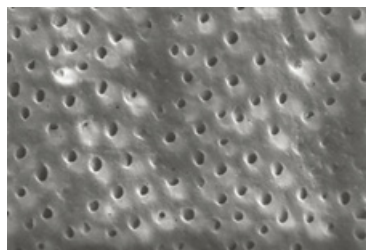(See Scanning Electron Micrographs: Figures 7a-9b)

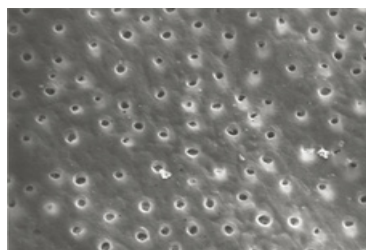**Figure7a:** Control specimen for Butler Protect (SEM X 3,000)

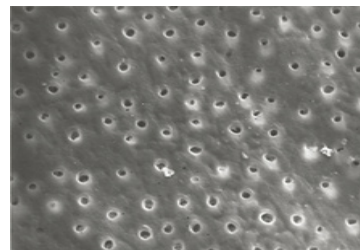
Figure 7b: Test specimen for Butler Protect (SEM X 3,000)

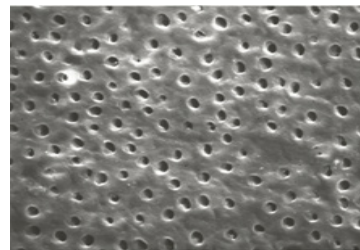**Figure 8a:** Control specimen for Macleans Sensitive (SEM X 3,000)

Figure 8b: Test specimen for Macleans Sensitive (SEM X 3,000)
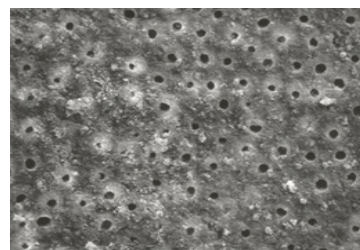

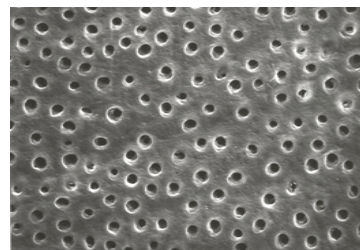**Figure 9a:** Control specimen for Colgate FluoriGard (Gel-Kam) (SEM X 3,000)

Figure 9b: Test specimen for Colgate FluoriGard (Gel-Kam) (SEM X 3,000)

Reproducibility of measurements
	The results of the repeated measurements were subjected to independent mean sampling t-tests and were observed to be not significantly different (Tables 3-4, 5a-5c). Results for the control and test specimens for each desensitizing agent were also subjected to independent mean sampling t-tests and these were also not significantly different.

Discussion

The hydraulic conductance of dentine is determined by several variables, which include the pressure moving fluid across the dentine, the length of the dentine tubules, the viscosity of the fluid and the radius of the tubule [12]. Clinical studies have shown that approximately 75% of the tubules are open in the sensitive areas of exposed dentine [13]. The number of patent tubules per unit area has been reported to be 8 times higher than in non-sensitive areas [8, 14]. The use of the dentine disc model for in vitro assessment of the potential efficacy of a desensitizing agent is therefore based upon the concept of tubule occlusion which according to the hydrodynamic theory will reduce tubule width and fluid movement within the tubule leading to subsequent desensitization. In this regard, the dentine disc provides a versatile and readily available model for DH studies. Its reliability however depends upon the precise location of the disc within the tooth and the precise positioning on the disc [10]. Despite the variations obtain among different human third molars, comparison between the two halves of the same disc prepared in a standardized manner can provide reasonably reliable results for the in vitro investigation.

The variations in dentine tubules in distribution, density, angles, width of the lumen, orientation, course of extension and branching, etc. makes the choice of the locations of any investigation crucial. Mjör and Nordhahl [15], in their studies of erupted premolars demonstrated such variations in different locations at different levels. The centre portion of the coronal dentine between the dentino-enamel junction (D.E.J.) and the pulp often provides, for in vitro assessment, the appropriate amount of dentine tubules with the same orientation and these tubules are often observed to be perpendicular to the viewing surface [16].

Previous studies assessing the tubular occluding effect of various desensitizing agents by viewing on dentine disc through SEM could only provide descriptive terms for example: partial, or complete blockage of tubules. Other investigators described their results in symbols, such as: +, ++, +++, etc. [16] or provided indices with percentages of occluded tubules [9,17]. Some of the previously described results were measured by means of a graticule [8] which may not be the most precise method. Although these investigators claimed that their method was quantitative in nature, these were mainly descriptive (qualitative) studies.

One of the interesting findings of the present study was the observation that desensitizing agents which were previously reported as having limited or little effect on the dentine surface [11], namely Potassium Oxalate and Gel-Kam, provided significant reduction in tubule width and area when analyzed by image analysis. This would appear to support the observation of Gillam et al. [18] who reported that descriptive or qualitative examination of the dentine surface, alone may be insufficient in the evaluation of the tubule occluding properties of selected desensitizing agents. One of the problems which still needs to be addressed in attempting to quantify the degree of tubule occlusion by image analysis is the ability of the operator (using the software package) to locate the precise outline of the tubule radius both pre and post assessment. At present due to the requirement of using different discs halves to assess the tubule occluding effect of the various desensitizing agents, it is possible despite adequate training that measurement error still exists. With the advent of more sophisticated Electron Microscopy technology and software packages such as ImageJ [19], however, it may now be possible to use the same disc as a control, take a photographic image, apply the desensitizing agent and take a second image and then compare the two negatives as demonstrated in the present study. By refining the procedure in this manner, it may then be possible to provide a superior method of quantifying the tubule occluding effects of desensitizing agents in the laboratory setting.

Conclusions

This study has demonstrated the usefulness of the image analysis system and SEM as a method for investigating and quantifying the effects of various treatments on dentine tubules in vitro. Furthermore, this method may also demonstrate differences between the tubule occluding properties of selected OTC or Professionally applied desensitizing agents.

References

1. Addy M, Mostafa P, Absi EG and Adams D. Cervical dentin hypersensitivity. Etiology and management with particular reference to dentifrices. In: Rowe, N.H. (ed): Proceedings of Symposium on Hypersensitive Dentin. Origin and Management, University of Michigan, (ed) E.& S. Livingstone Limited, Edinburgh & London 1985; 147-167.

2. Addy M and Urquhart E. Dentine hypersensitivity: its prevalence, aetiology and clinical management. Dental Update Dec 1992; 19(10):407-8, 410-412.

3. Gillam DG. The assessment and treatment of cervical dentinal sensitivity. DDS thesis, 1992; University of Edinburgh, United Kingdom.

4. Ong G. Desensitizing Agents. A review. Clin Prev Dent 1986; 8, 14-18.

5. Scherman A and Jacobsen PL. Managing dentin hypersensitivity: What treatment to recommend to patients. J Am Dent Assoc 1992; 123, 57-61.

6. Gillam DG Mechanisms of stimulus transmission across dentin - a Review. Periodontal Abstr 1995; 43(2): 53-65.

7. Brannstrom M and Arne Astrom. A hydrodynamic mechanism in the transmission of pain producing stimuli through the dentine, In: Sensory Mechanism in Dentine. Anderson, D.J. (ed) Oxford Pergammon Press 1963;73-79.

8. Absi EG, Addy M and Adams D. Dentine hypersensitivity: A study of the patency of dentinal tubules in sensitive and non-sensitive cervical dentine. J Clin Periodontol 1987; 14(5): 280-284.

9. McAndrew R. and Kourkouta S. Effects of toothbrushing prior and/or subsequent to dietary acid application on smear layer formation and the patency of dentinal tubules: A SEM study. J Periodontol 1995; 66: 443-448.

10. Mordan NJ, Barber PM and Gillam DG. The dentine disc. A review of its applicability as a model for the in vitro testing of dentine hypersensitivity. J Oral Rehabil. 1997; 24(2):148–156.

11. Ling TYY, Gillam DG, Barber PM, et al. An investigation of potential desensitizing agents in the dentine disc model: a scanning electron microscopy study. J Oral Rehabil 1997; 24(3): 191-203.

12. Pashley DH. Mechanism of dentin sensitivity. Dent Clin N Amer 1990; 34, 449-473.DH.

13. Yoshiyama M, Masada J, Uchida A, et al. Scanning electron microscopic characterization of sensitive vs. insensitive human radicular dentin. J Dent Res 1989; 68(11): 1498-1502.

14. Absi EG, Addy M and Adams D. Dentine hypersensitivity: The development and evaluation of a replica technique to study sensitive and non-sensitive cervical dentine. J Clin Periodontol 1989; 16(3): 190-195.

15. Mjör I.A.and Nordahl I. The density and branching of dentinal tubules in human teeth. Arch Oral Biol 1986; 41(5): 401-412.

16. Absi EG, Addy M and Adams D. Dentine hypersensitivity: uptake of toothpastes onto dentine and effects of brushing, washing and dietary acid - SEM in vitro study. J Oral Rehab 1995; 22: 175-182.

17. Rimondini L, Baroni C and Carrassi A. Ultrastructure of hypersensitive and non-sensitive dentine. J Clin Periodont 1995; 22(12): 899-902.

18. Gillam DG, Mordan NJ and Newman HN. The Dentin Disc Surface: A Plausible Model for Dentin Physiology and Dentin Sensitivity Evaluation. Adv Dent Res 1997; 11(4):487–501.

19. Schindelin J, Rueden CT, Hiner, MC, et al. "The ImageJ ecosystem: An open platform for biomedical image analysis", Molecular Reproduction & Development 2015; 82(7-8):518–529.