Mestrado Integrado em Medicina Dentária

Faculdade de Medicina da Universidade de Coimbra

Avaliação ultramorfológica e química das superfícies dentinárias resultantes da ação de duas pastas dentífricas: estudo *in vitro*

Ultramorphological and chemical evaluation of dentinal surfaces formed following application of two different toothpastes: an *in vitro* study

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Ultramorphological and chemical evaluation of dentinal surfaces formed following application of two different toothpastes: an *in vitro* study

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Abstract

Introduction: Dentin hypersensitivity is considered one of the most prevalent painful condition of the oral cavity, but poorly understood and is defined as a short, sharp pain arising from exposed dentin in response to stimuli typically thermal, evaporative, tactile, osmotic or chemical presenting, many times, a multifactorial etiology.

Purpose: The aim of this in vitro study was to evaluate the obliteration of dentinal tubules after application of two different dentifrices using scanning electron microscopy for ultra structural analysis and energy dispersive X-ray spectroscopy (EDX) for chemical evaluation of a selected area.

Materials and Methods: Five extracted human third molars were collected, stored at room temperature in a 10% buffered formalin solution (pH=7.0) for up to 3 months. Five dentin discs were obtained by sectioning each tooth parallel to the occlusal surface from the top of the pulp horns and occlusally to an approximate 1 mm. After each dentin disc was sectioned into four quarters, they were carefully and singly stored in artificial saliva until required. All surface specimens were etched with application of acid citric 6% for 2 minutes. The specimens were equally distributed into four groups each containing five samples and different treatments: control (G1; without any treatment), artificial saliva (G2) and two toothpastes: Oral-B® Pro Expert (G3) and Sensodyne® Repair&Protect™ (G4). In G2, 3 and 4 surface samples were brushed twice-daily during 14 days. Specimens from each group were SEM and EDX examined. Tubule occlusion score was statistically analyzed and intergroup compared and chemical analyses was also made to evaluate differences between groups.

Results: At 750-fold magnification the score for tubule occlusion for the control group (group 1) was the lowest (1,6±0,548) and the Sensodyne® Repair&Protect™ group (group 4) had the highest one (4,8±1,095). Artificial saliva group (group 2) and Oral-B® Pro Expert group (group 3) had, respectively, (3±1,414) and (4,2±1,304). Intergroup comparison revealed statistically significant differences ($p<0{,}05$; C.I. 95%) between group 1 and group 2 ($p=1{,}016$), group 1 and group 4 ($p=0{,}007$) and group 2 and group 4 ($p=0{,}042$).

The EDX chemical analysis of the occlusion deposits revealed, for group 1, high levels of carbon, oxygen and nitrogen and lower levels of calcium and phosphorus. For group 2 an increase of the levels of the phosphorous and calcium elements and a simultaneous drop of oxygen and carbon levels were found. Group 3 results demonstrated a surface layer mainly composed of calcium and phosphorous with additional signs of silicon, zinc and sodium. Also, group 4 had occlusion deposits predominantly composed of calcium and phosphorous but additional discrete signs of titanium and sodium were also found.

Conclusions: Within this *in vitro* study limitations it is possible to say that Sensodyne® Repair&Protect™ demonstrated the highest mean score recording the level of occlusion for dentin tubule and the most consistent results in final score distribution.

Keywords: Dentin hypersensitivity, dentin tubules, scanning electron microscopy, energy dispersive X-ray, toothpaste

Resumo

Introdução: A hipersensibilidade dentinária é considerada uma das condições mais dolorosas prevalente na cavidade oral, apesar de ser ainda pouco

compreendida. É definida como uma dor aguda e curta proveniente da dentina exposta ao meio oral em resposta a um estímulo térmico, volátil, tátil, osmótico ou químico apresentando, muitas vezes, uma etiologia multifactorial.

Objectivos: O objetivo deste estudo *in vitro* foi avaliar a obliteração dos túbulos dentinários após a aplicação de duas diferentes pastas dentífricas. Amostras de ambos grupos foram analisadas através de microscopia eletrónica de varrimento (MEV) espectroscopia de raios X por dispersão em energia (EDX) para avaliação química de uma área selecionada. A oclusão dos túbulos dentinário foi estatisticamente analisado e procedeu-se à comparação intergrupos.

Materiais e métodos: Cinco dentes humanos íntegros, 3ºmolares, foram extraídos e armazenados à temperatura ambiente em 10% de solução de formaldeído (pH=7,0) por um período de tempo até 3 meses. Cinco discos de dentina com uma espessura aproximada de 1mm foram obtidos seccionando cada dente paralelamente à superfície oclusal e à parte superior dos cornos pulpares. De seguida, foram cortados cuidadosamente em quatro quartos e armazenados separadamente em saliva artificial até serem necessários. Todas as superfícies das amostras foram sujeitas a um condicionamento com a aplicação de ácido cítrico a 6%, durante 2 minutos. Os discos foram distribuídos igualmente em quatro grupos, cada um contendo cinco discos e posteriormente sujeitos a tratamentos: controlo (G1, sem qualquer tratamento), saliva artificial (G2) e duas pastas dentífricas: Oral-B® Pro Expert (G3) and Sensodyne® Repair&Protect^{TM} (G4). A superfície das amostras dos G2, 3 e 4 foram escovadas 2 vezes ao dia durante 14 dias. As amostras de cada grupo foram observadas em MEV e EDX. A oclusão dos túbulos dentinários foi estatísticamente analisada e a análise química foi igualmente realizada para avaliar as diferenças entre os grupos.

Resultados: Na ampliação 750 a pontuação para a oclusão dos túbulos para o grupo controle (grupo 1) foi a mais baixa (1,6 ± 0,548) e o grupo Sensodyne® Repair&Protect^{TM} (grupo 4) teve a mais elevada (4,8 ± 1,095). O grupo saliva artificial (grupo2), e o grupo Oral-B® Pro Expert (grupo 3) apresentaram, respetivamente, (3 ± 1,414) e (4,2 ± 1,304). A comparação entre os grupos revelou diferenças estatisticamente significativas (p <0,05, IC 95%) entre os grupos 1 e 2 (p = 1,016), entre o grupo 1 e o grupo 4 (p = 0,007) e entre o grupo 2 e grupo 4 (p = 0,042).

A análise química (EDX) da superfície oclusal, revelou para o grupo 1, elevados níveis de carbono, oxigénio e azoto e níveis mais baixos de cálcio e fósforo. Para o grupo 2 verificou-se um aumento dos níveis de fósforo e de cálcio e uma diminuição dos níveis de oxigénio e de carbono. No grupo 3 os resultados demonstraram uma camada superficial composta principalmente por cálcio e fósforo com sinais adicionais de silício, zinco e sódio. Também no grupo 4 foi possível observar uma camada superficial predominantemente composta por cálcio e fósforo e adicionais sinais discretos de titânio e sódio.

Conclusões: Dentro das limitações inerentes a este estudo *in vitro* podemos afirmar que a pasta dentífrica Sensodyne® Repair&Protect™ apresentou a maior média de pontuação para o nível de oclusão de túbulos dentinário bem como os resultados mais consistentes na distribuição da pontuação final.

Introduction

Dentin hypersensitivity (DH) is defined as a short, sharp pain arising from exposed dentin in response to stimuli typically thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other form of dental defect or pathology.[1] The discomfort or pain of dentin hypersensitivity can be unpleasant and bothersome. However, in severe cases, it may have a significant negative impact on an individual's daily life, as it may cause difficulties in eating and drinking, especially items with hot and cold temperatures and it may even interfere with speaking.[2]

Clinical surveys showed that the prevalence of dentin hypersensitivity ranged from 2.8% to 74%, depending on the involved population, study setting and study *design.*[3] This pathology mostly affects individuals at their fourth and fifth decade of life.[4] Epidemiological surveys indicate that the majority of sensitive surfaces are the buccal-cervical margins with a higher incidence in the pre-molars area. Nevertheless, other surfaces may also be affected as any site of exposed dentin may exhibit sensitivity, but not all exposed dentin is sensitive.[5,6] Due to its high prevalence in the general population and its impact on patients, significant efforts have been made to understand the etiology and mechanisms involved in the development of dentin hypersensitivity.[7] Several factors were identified as being involved in dentin hypersensitivity development, among them: gingival recession; periodontal disease; deep tooth cracks and loss of enamel, cementum, and dentin due to mechanical abrasion, chemical erosion, and tooth fracture.[8,9]

Regardless of the etiology of dentin exposure, a common feature of hypersensitive dentin is the presence of open dentin tubules, which provide a direct link between the external environment and the pulp of the tooth. Several theories to explain dentine hypersensitivity have been proposed but the most widely accepted is the hydrodynamic theory proposed by Brannstrom.[10] This concept suggested that pain may result from the movement of the dentin fluid in the tubules induced by external stimuli which, in turn, trigger nerve fibers within the pulp resulting in pain.[11] *In vivo* studies revealed that the response of the pulpal nerves was proportional to the pressure and therefore to the rate of fluid flow.[12-14]

There are a large number of treatment options for managing dentinal hypersensitivity using chemical or physical agents. Current treatments tend to concentrate on two approaches: tubule occlusion or neural transmission blockage. The majority of treatment methods attempt to inhibit sensitivity by occluding the dentinal tubules. This may be achieved either by altering their contents, or by creating insoluble calcium complexes, thus forming mechanical or chemical plugs.[15] The most common form to produce the above described effect is the placement of a topically applied agent enforced either by a dental professional or by the patient at home.

Overall, patient responses are very subjective and thus treatment results are largely dependent upon the individual's pain threshold.[7] Several chemical agents have been employed for this purpose, such as, strontium chloride, sodium fluoride, stannous fluoride, potassium oxalate, potassium nitrate among others. Molecules based on potassium anions acts by reducing the sensory activity and their mode of action lies on the depolarizing activity of the potassium ion due to the increase in its extracellular concentration around the nerves deep in dentin making them less excitable and also by preventing the repolarization of the nerve.[16] Nevertheless, most of the therapies proposed to date for DH treatment rely on the sealing off the dentinal tubules. Recently, two new promising molecules have been incorporated in different dentifrices for hypersensitivity management: calcium sodium phosphosilicate (CSPS), commercially known as NovaMin and a stabilized stannous fluoride dentifrice containing sodium hexametaphosphate (SFSH).

Calcium sodium phosphosilicate is an inorganic amorphous compound that contains only calcium, sodium, phosphate and silica.[17] When CSPS particles comes into contact with an aqueous environment such as water or saliva, an immediate release of sodium ions occur, which increases the local environment pH. The surface reactions include the ion exchange between Na^{2+} from CSPS and H^+ from the fluid resulting in the formation of a porous silica rich layer on the surface which provides a nucleating site for the rapid and early precipitation of a calcium phosphate hydroxycarbonate apatite layer.[18,19] Damen and ten Cate *et al*[20] studied the effect of soluble silica on the precipitation of calcium phosphates mineral and have demonstrated that polymers of silicic acid increased the rate of precipitation of hydroxyapatite, even in the presence of inhibitors of hydroxyapatite.

Since the early 1950s that stannous fluoride have been incorporated in dental dentifrices and there is considerable evidence for its efficacy as a therapeutic agent with a wide spectrum of beneficial properties as it can provide protection against caries, pathogenic bacteria, gingivitis, hypersensitivity and plaque development.[21,22] However, its clinical usage was limited because of astringent taste, and in some patients, its use resulted in extrinsic staining of the teeth. Those limitations were outdated when a novel dentifrice formulation was introduced combining stabilized stannous fluoride, sodium hexametaphosphate, and silica (SFSH). This formula was introduced offering the therapeutic benefits of a 0.454% stabilized stannous fluoride with the calculus and stain-control characteristics of sodium hexametaphosphate in a low-water formulation dentifrice.[23] When this anhydrous preparation is applied on dentin surfaces the occlusion of tubules by a tin-rich low solubility complexes is expected.[22]

The aim of this *in vitro* study was to evaluate the obliteration of dentinal tubules after application of two different dentifrices Oral-B Pro Expert and Sensodyne Repair&Protect using scanning electron microscopy (SEM) for ultra structural analysis and energy dispersive X-ray spectroscopy (EDX) for chemical evaluation of a selected area. The null hypothesis is that there would be no differences regarding dentinal tubules obliteration between the tested materials.

Materials and methods

Dentin sample preparation

Five caries free, intact and freshly extracted human third molars were collected after the patient`s informed consent, as approved by the Medical Faculty Ethical Committee. The teeth were cleaned from all surrounding soft tissues and stored at room temperature in a 10% buffered formalin solution (pH 7.0) for up to 3 months after extraction and until used in the experiment. Five dentin discs were obtained by sectioning each tooth parallel to the occlusal surface from the top of the pulp horns and occlusally to an approximate 1 mm width using a hard tissues cutting saw (Accutom 50, Struers, Ballerup, Denmark) with water as coolant. Each disc was then sectioned with a diamond disc mounted in a high speed hand piece into four quarters whilst the discs were kept moist. A small area was dried on each section at the upper coronal surface and marked for identification with one dot in the side unstudied in which a stick was glued to facilitate the continuity of the experimental design (figure 1 e 2). Every four specimens obtained from one dentin disc were carefully and singly stored in artificial saliva until required. This procedure allowed for strict comparisons to be made between group, and the marked samples could be easily identified and oriented for examination.

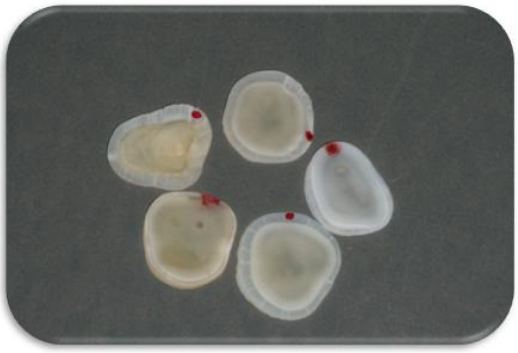


Fig. 1: Five dentin discs marked with a dot in the unstudied side.

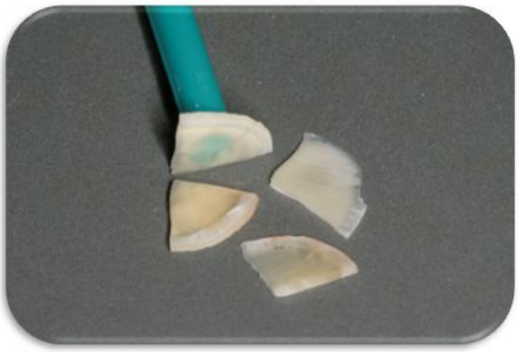


Fig. 2: Dentin disc sectioned into four quarters held by a stick.

Experimental design

Initially, all specimens had their smear layer removed by ultrasonication in deionized water for 30 seconds followed by etching the surface with application of 6% citric acid for 2 minutes and rinsing with distilled water for 30 seconds.[24] This procedure was necessary to create open tubules and simulate clinical conditions similar to those found on dentine hypersensitivity. The specimens were equally distributed into four groups each containing five samples, according to one of the following protocols (Table 1):

Group 1 (Control) (n=5): Etched specimens were immersed in artificial saliva without any treatment, for 14 days.
Group 2(n=5): Etched specimens were brushed with artificial saliva for 30 seconds twice daily for 14 days.
Group 3(n=5): Etched specimens were brushed with Oral-B® Pro Expert (Procter & Gamble UK, Weybridge, UK) for 30 seconds twice daily for 14 days.
Group 4(n=5): Etched specimens were brushed with Sensodyne® Repair&Protect (GlaxoSmithKline; Slough, UK) for 30 seconds twice daily for 14 days.

Table1: Artificial saliva and toothpastes composition.

Materials	Composition (mg/1,000 ml)	Lot nr.
Artificial saliva	Nacl 125.6,KC 963.9,CaCl2.2H2O 227.8, KH2PO4 654.5,Urea 200.0, NH4Cl 178.0, NaHCO3 630.8, KSCN 189.2, Na2SO4.10H2O 763.2	
Oral-B® Pro Expert Procter & Gamble UK, Weybridge, UK	Glycerin, Hydrated Silica, Sodium Hexametaphosphate, Propylene Glycol, PEG-6, Aqua, Zinc Lactate, Sodium Lauryl Sulfate, Aroma, Sodium Gluconate, Chondrus Crispus Powder, Trisodium Phosphate, Stannous Fluoride, Sodium Saccharin, Xanthan Gum, Copernicia Cerfera Cera, Cinnamal, Silica, Sodium Fluoride, cl 77891, Eugenol, cl 74160. Fluor(1450ppm)	GGC8
Sensodyne® Repair&Protect (GlaxoSmithKline; Slough, UK)	Glycerin, Silica, Calcium Sodium Phosphosilicate (NovaMin), Sodium Lauryl Sulfate, Sodium Monofluorophosphate, Aroma, Titanium Dioxide, Carbomer, Potassium Acesulfame, Limonete, Fluor (1450ppm).	152D G1

A single-tuft toothbrush mounted in an electric brushing device (Oral-B® Professional Care® 500, Procter & Gamble Co., Cincinnati, OH, USA) was applied at a 90º inclination to the dentin surface, under a constant loading for 30 seconds (Figure 3).

In group 2, samples were brushed with 4 ml of artificial saliva, whilst in group 3 and 4 specimens were brushed with 40 g of the tested toothpaste without any dilution. Samples from all these groups were brushed during 30 seconds before being gently rinsed with 10 ml of deionized water for 10 seconds and stored in artificial saliva at 37ºC, until used in the next brushing session. Artificial saliva was changed after all protocolled brushing periods.

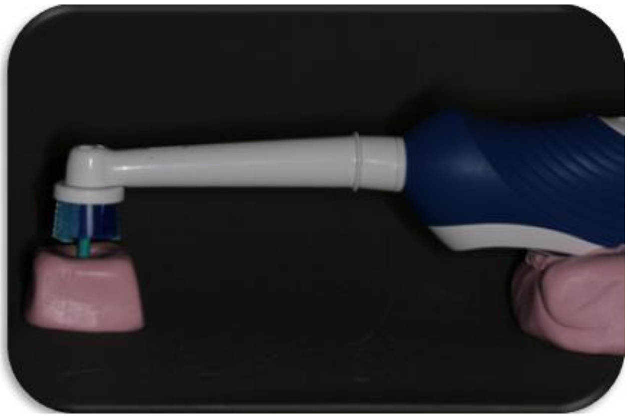


Fig. 3: A single-tuft toothbrush linked in an electrical toothbrushing machine providing a 90º inclination to the dentin surface.

SEM analysis

For SEM analysis all samples were first fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48h at room temperature. The specimens were then dehydrated in graduated alcoholic solutions and submitted to chemical drying in hexamethyl disilazane (HMDS) until its complete evaporation, and then covered with an absorbent paper. All samples were mounted on aluminium stubs using carbon sticky pads, sputter coated with gold and subjected to analysis in a scanning electron microscope (JSM 5310 , JEOL; Tokyo, Japan) (figure 4). The acceleration voltage was set to 10 kV. After this procedure, one representative specimen of each group was fractured into halves to achieve a cross section located perpendicular to the border allowing SEM visualization of the depth of the occluded tubules.

Additionally, energy dispersive X-ray (EDX) analysis was performed from two surface samples of each group. The acceleration voltage of the scanning electron microscope was set to 20 kV and EDX spectra were collected using a Si-detector (X-Max^{N} detector, Oxford Instruments, Oxfordshire, UK). Spectra were then processed using AZtecEnergy analysis software (AZtec, Oxford Instruments, Oxfordshire, UK) for surface element composition detection and for relative element contents calculation in weight percentage (wt.%).

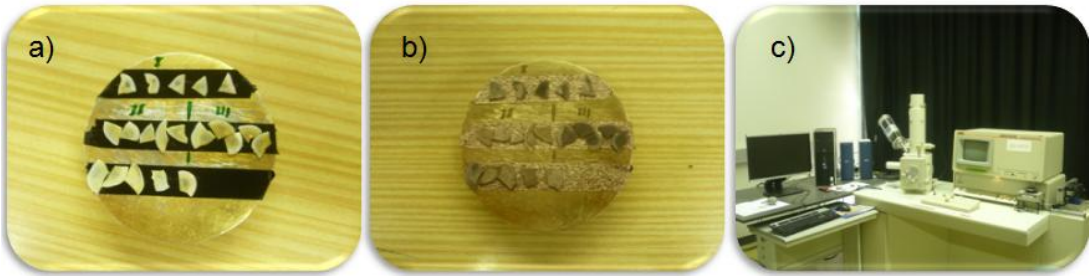


Figure 4: Dentin samples: a) mounted in an aluminium stub; b) sputter-coated with gold; c) analysed by SEM and EDX.

One representative photomicrograph was taken from each studied dentinal surface at a 750-fold and 2000-fold magnification to assess the level of tubule occlusion. Image grading was performed based on those micrographs by two independent evaluators blinded to the study based on a six-point scale (figure 5):

Score 1: open tubules; completely unoccluded;
Score 2: most tubules open (~90%);
Score 3: tubules are occluded around 50%;
Score 4: most tubules occluded, tubules outlines visible;
Score 5: most tubules occluded (~90%);
Score 6: all tubules occluded.

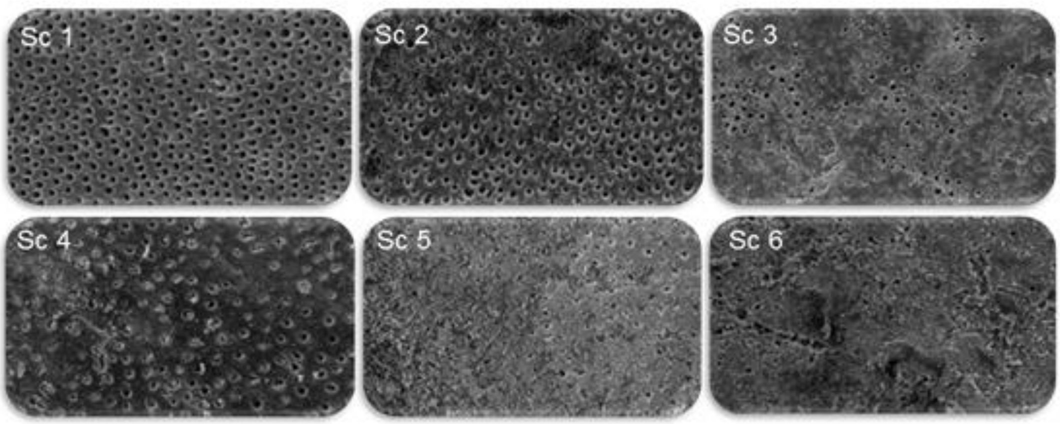
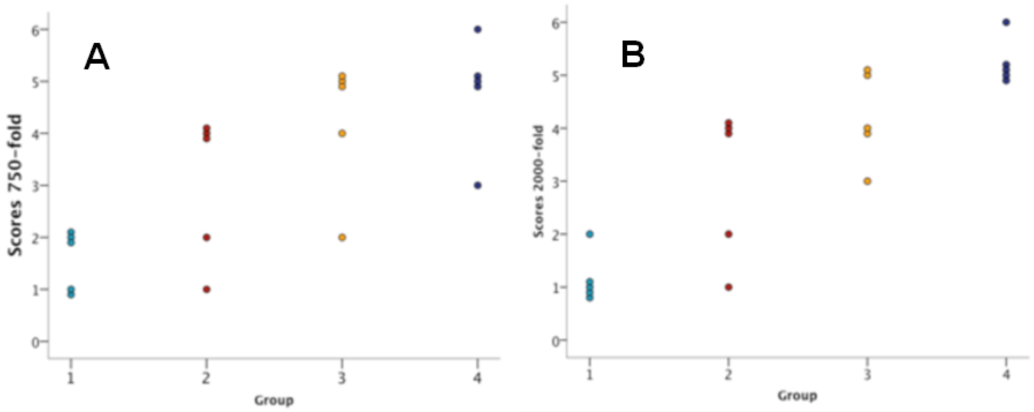


Figure 5: Representatives micrographs for the six-point occlusion scale. Sc: Score; Score 1; Score 2; Score 3; Score 4; Score 5; Score 6.

Results

The statistical analysis of the data was performed using IBM® SPSS Statistics Version 20.0 for Mac (SPSS, Chicago, IL, USA).

Two independent blinded examiners observed the 750-fold and 2000-fold magnification samples separately. The samples were classified according to the pre-established scores. After obtaining the results from each examiner the between-observers agreement was quantified by intraclass correlation coefficient analysis for single measures for (ICC= 0,937, *p*<0,01). Despite the very good agreement, the two examiners got to consensus on the discrepant cases for statistical analysis execution (Graphic 1).



Graphic 1: Score distribution according groups: 750-fold (A) and 2000-fold (B) magnification.

Normality of the data distribution was violated as assessed by the Shapiro-Wilk test (p<0.01). Thus, non-parametric group comparison was performed using Kruskal-Wallis and Mann-Whitney as post-hoc pairwise comparison test. Wilcoxon signed-ranks test was applied for intragroup comparison at 750-fold and 2000-fold magnifications. Significance level was set at 0.05.

Table 2 represents the descriptive statistics the group scores for the 750-fold magnification (A) and for 2000-fold magnification (B). The control group (group1) revealed the lowest mean score (1,6±0,548) indicating the least percentage of tubules occlusion while the NovaMin^{TM}-based toothpaste (group4) demonstrated the highest mean score (4,8±1,095) recording the level of occlusion and the most consistent results in final score distribution.

Table 2 - Tubules occlusion score values for a 750-fold magnification and 2000-fold magnification (Mean± Standard deviation) (Confidence Interval for mean [95% CI]).

	Groups				Inter-group Comparison				
	Control(1)	Saliva (2)	OralB® Pro Expert (3)	Sensodyne® Repair& Protect (4)	*P* value				
	n=5	n=5	n=5	n=5	1 *vs* 2	1 *vs* 3	1 *vs* 4	2 *vs* 3	2 *vs* 4
X750	1,6±0,548 [0,92 ; 2,28]	3±1,414 [1,24 ; 4,76]	4,2±1,304 [2,58 ; 5,82]	4,8±1,095 [3,44 ; 6,16]	p>0,05 (*NS*)	p=0,016 (*SS*)	p=0,007 (*SS*)	p>0,05 (*NS*)	p=0,042 (*SS*)
	n=5	n=5	n=5	n=5	1 *vs* 2	1 *vs* 3	1 *vs* 4	2 *vs* 3	2 *vs* 4
X2000	1,2±0,447 [0,64 ; 1,76]	3±1,414 [1,24 ; 4,76]	4,2±0,837 [3,16 ; 5,24]	5,2±0,447 [4,64 ; 5,76]	p=0,041 (*SS*)	p=0,007 (*SS*)	p=0,005 (*SS*)	p>0,05 (*NS*)	p=0,006 (*SS*)

SS: statistically significant (P≤0,05); NS: not statistically significant (P>0,05); *vs: versus* .

Statistical between-treatment comparisons of the occlusion scores mean ranks for 750-fold and 2000-fold magnification revealed significant differences between the studied groups (Kruskal-Wallis, p=0.009).

Post-hoc Mann-Whitney was applied for analysis of two independent samples for 750-fold and 2000-fold magnification.

For 750-fold magnification statistically significant differences were found between control group (group 1) and both Oral-B & Pro-Expert® (group 3) (*p*=0,016) and Sensodyne Repair & Protect® (group 4) (*p*=0,007) and Sensodyne Repair & Protect® (group 4) was also significantly different from artificial saliva (group 2) (*p*=0,042).

For 2000-fold magnification statistically significant differences were found between control group (group 1) and both Oral-B & Pro-Expert® (group 3) (*p*=0,007) and Sensodyne Repair & Protect® (group 4) (*p*=0,005). Sensodyne Repair & Protect® (group 4) was also significantly different from artificial saliva (group 2) (*p*=0,006). Statistically significant differences were found between control group (group 1) and artificial saliva (group 2) (*p*=0,041) and between Oral-B & Pro-Expert® (group 3) and Sensodyne Repair & Protect® (group 4) (*p*=0,045).

At both magnifications no statistical differences were found between any other pair of groups.

Wilcoxon signed ranks test showed no differences between scores obtained at the 750-fold and the 2000-fold magnification (*Z*= -0,087, *p*=0,931).

All samples of the demineralized control group (group 1) exhibited open dentin tubules as it was expected (figure 6 A, B). From cross-sectional view it could be noticed that demineralization depth could reach 20 μm (figure 7). The EDX chemical mapping of the respective dentine surface showed high levels of carbon, oxygen and nitrogen and lower levels of calcium and phosphorus (figure 8). Relative element contents calculation in weight percentage (wt.%) obtained are shown in table 3.

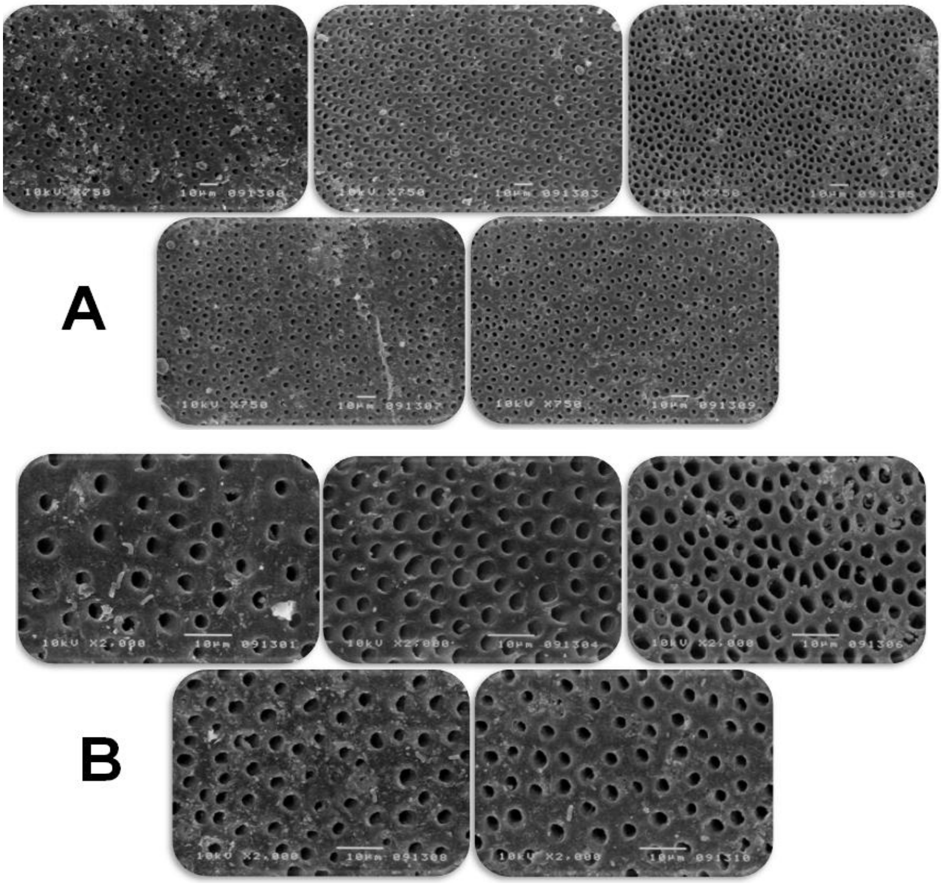


Figure 6: Representative photomicrographs of surface dentin samples kept in artificial saliva (control group) without brushing after fourteen days storage at a 750-fold (A) and 2000-fold (B) magnification.

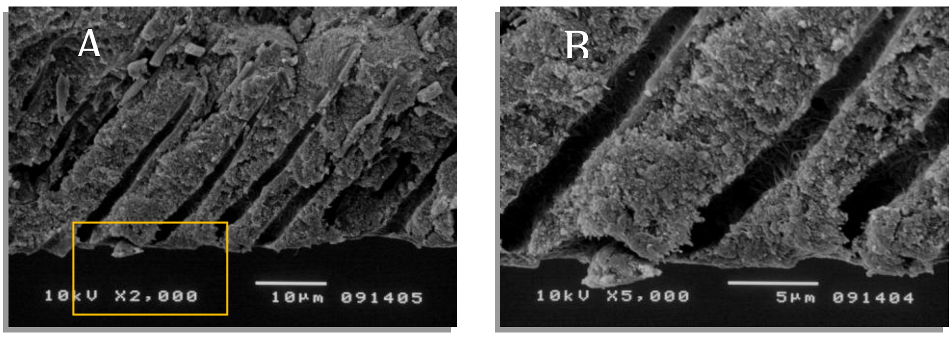


Figure 7: Representative photomicrographs of cross-sectioned dentin samples kept in artificial saliva (control group) after fourteen days storage at a 2000-fold (A) and 5000-fold (B) magnification. Open tubules running from the surface are evident.

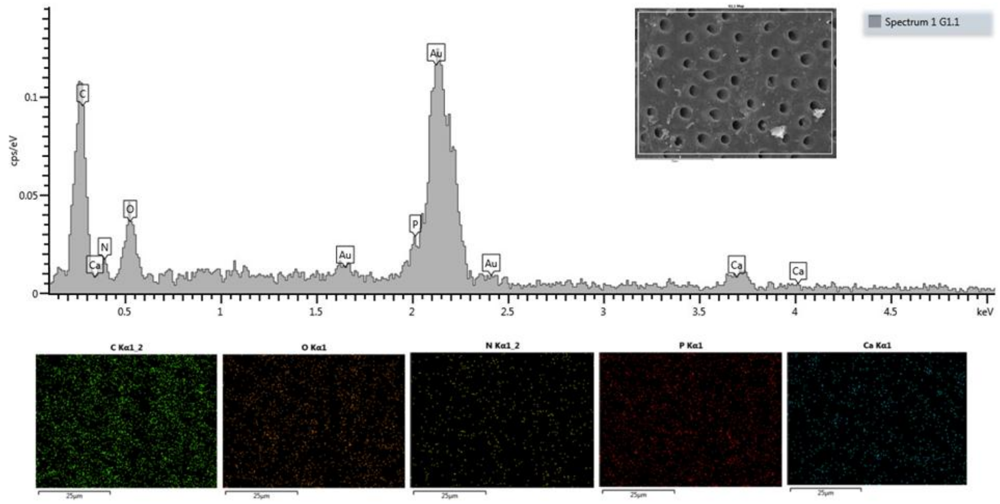


Fig. 8: Energy dispersive X-ray spectroscopy (EDX) spectra from a representative sample of group 1 showing a high levels of the carbon and oxygen elements.

Table 3: Relative element contents calculation in weight percentage (wt.%) for each group obtained from EDX analysis

Spectrum Labe	C	N	O	Na	Si	P	Ca	Ti	Zn	Total
G1	83.29	7.49	9.08			0.03	0.12			100.0
G2	16.09		44.75			9.04	30.12			100.0
G3	6.97		19.09	0.71	4.14	14.38	51.92		2.80	100.0
G4	11.59		30.94	0.84		17.64	38.33	0.66		100.0

In group 2, samples revealed a reduction in the tubule lumen diameter and a considerable number of tubules obliterated, while maintaining tubules outlining mostly visible (figure 9 A, B). From cross-sectional view it could be noticed that its entrance was occasionally filled with precipitates with no more than 2 μm depth (figure 10). Chemical mapping showed an increase of the levels of the phosphorous and calcium elements and a simultaneous drop of carbon levels (figure 11 and table 3).

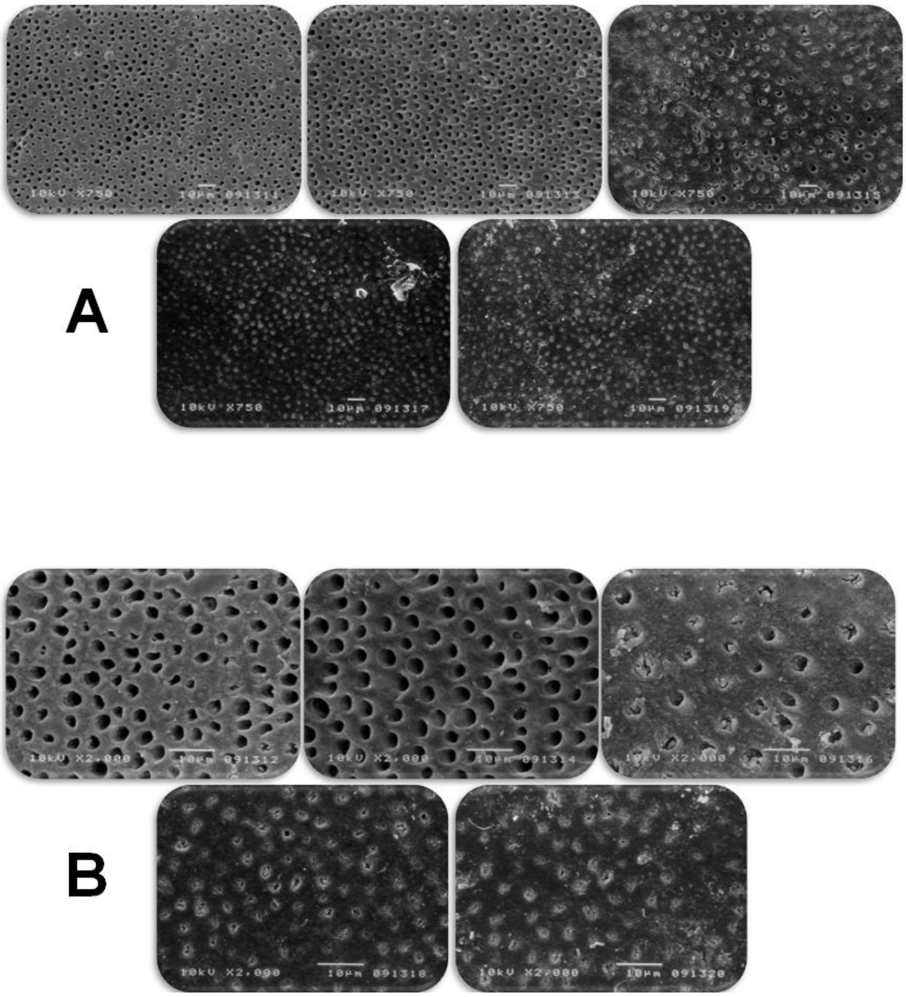


Figure 9: Representative photomicrographs of surface dentin samples brushed with artificial saliva (group 2) without any toothpaste after fourteen days treatment at a 750-fold (A) and 2000-fold (B) magnification. On the surface it can be observed a reduced.

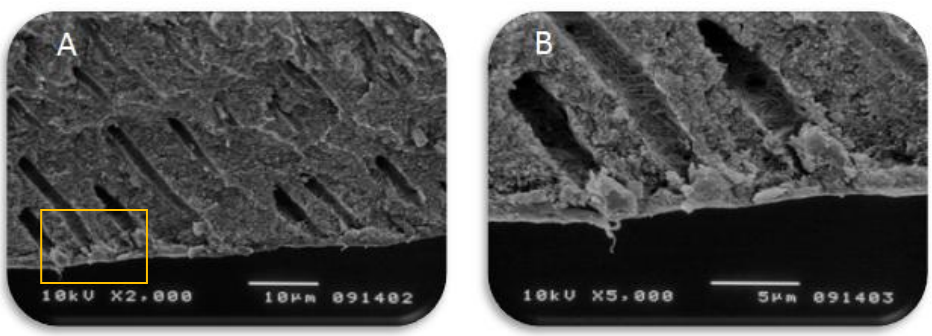


Figure 10: Representative photomicrographs of cross-sectioned dentin samples brushed with artificial saliva (group 2) without any toothpaste after fourteen days at a 2000-fold (A) and 5000-fold (B) magnification. Discrete precipitations can be observed inside the entrance of dentin tubules.

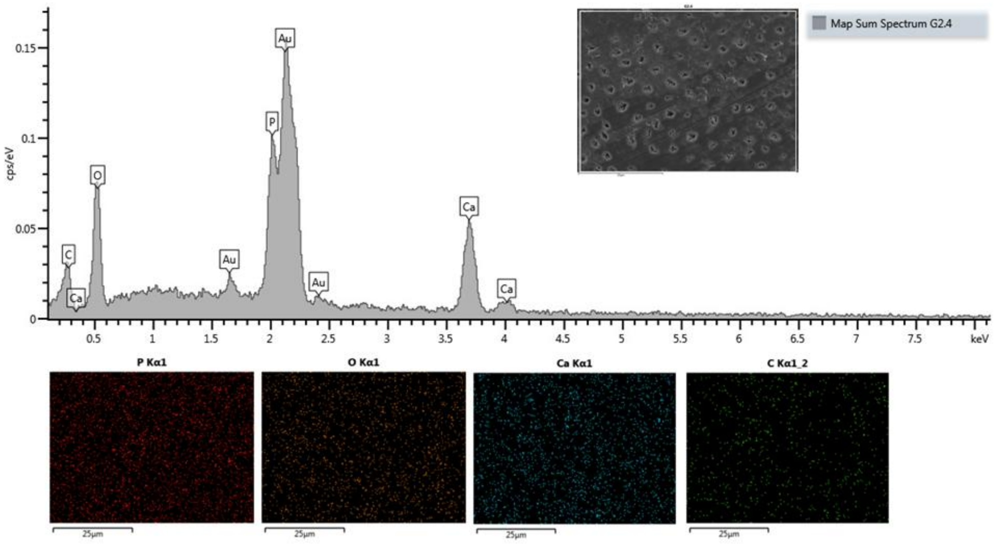
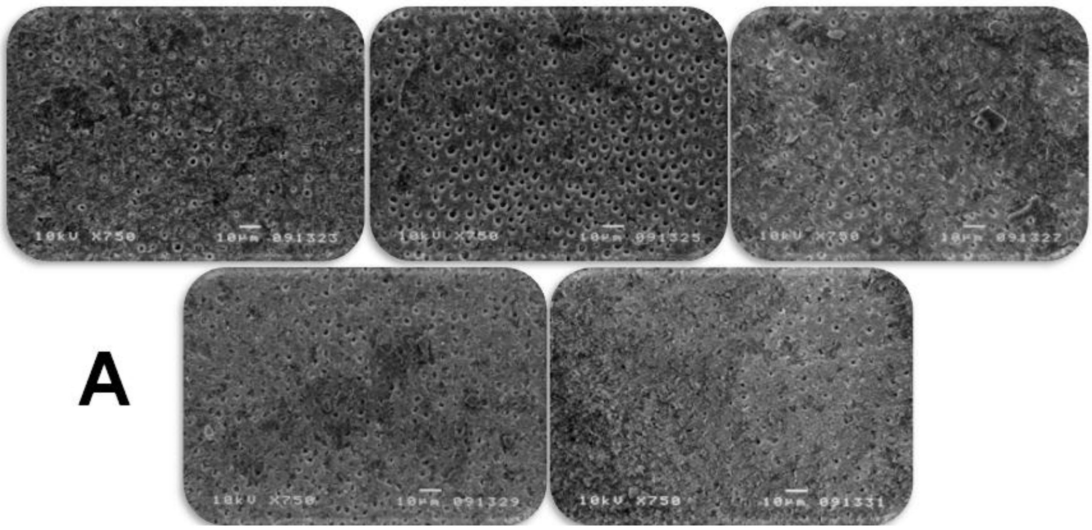


Figure 11: Energy dispersive X-ray spectroscopy (EDX) spectra from a representative sample of group 2 showing high levels of the phosphorus and calcium elements.

Group 3 treated dentin surfaces presents an incomplete surface coating layer where a great number of dentinal tubules became partially or completely obliterated (figure 12 A, B). From cross-sectional view it could be noticed a generalized reduction in tubule diameter and precipitates occluding tubule lumens (figure 13). EDX chemical map taken alongside a secondary electron image of a mapped area showed that the surface layer is mainly composed of calcium and phosphorous.

Additionally, signs of silicon, zinc and sodium were also found (figure 14 and table 3).



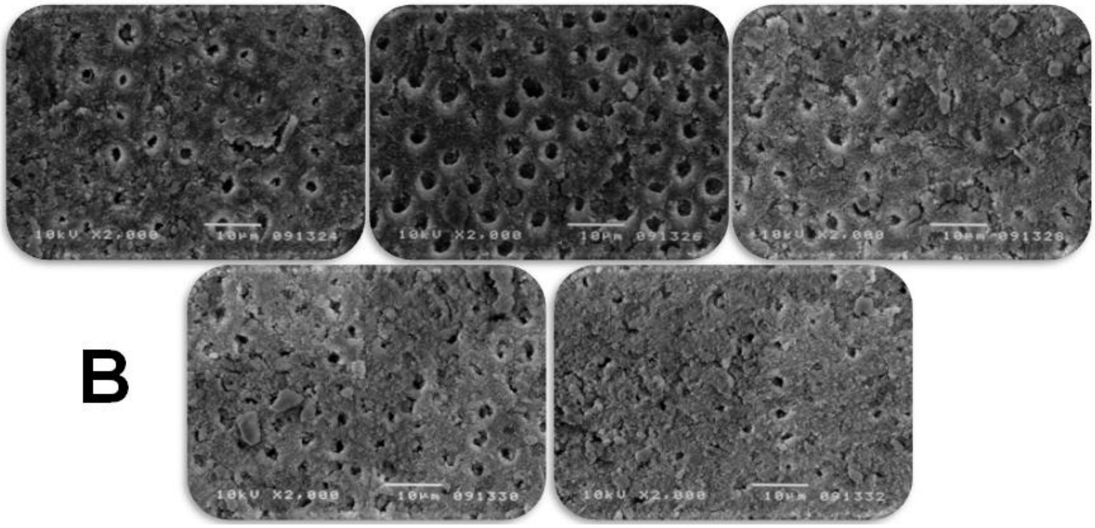


Figure 12: Representative photomicrographs of surface dentin samples brushed with Oral-B® Pro Expert (group 3) after fourteen days treatment at a 750-fold (A) and 2000-fold (B) magnification. On the surface it can be observed an incomplete coating layer covering partially or completely tubule lumen.

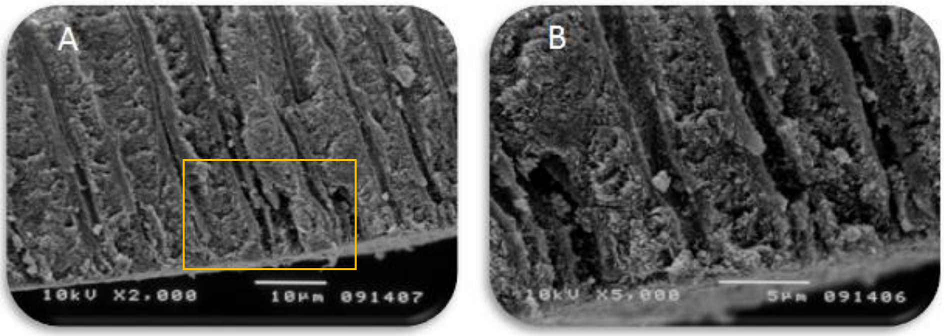


Figure 13: Representative photomicrographs of cross-sectioned dentin samples samples brushed with Oral-B® Pro Expert (group 3) after fourteen days at a 2000-fold (A) and 5000-fold (B) magnification. From this view it can be observed a generalized reduction in tubule diameter and precipitates occluding tubule lumen.

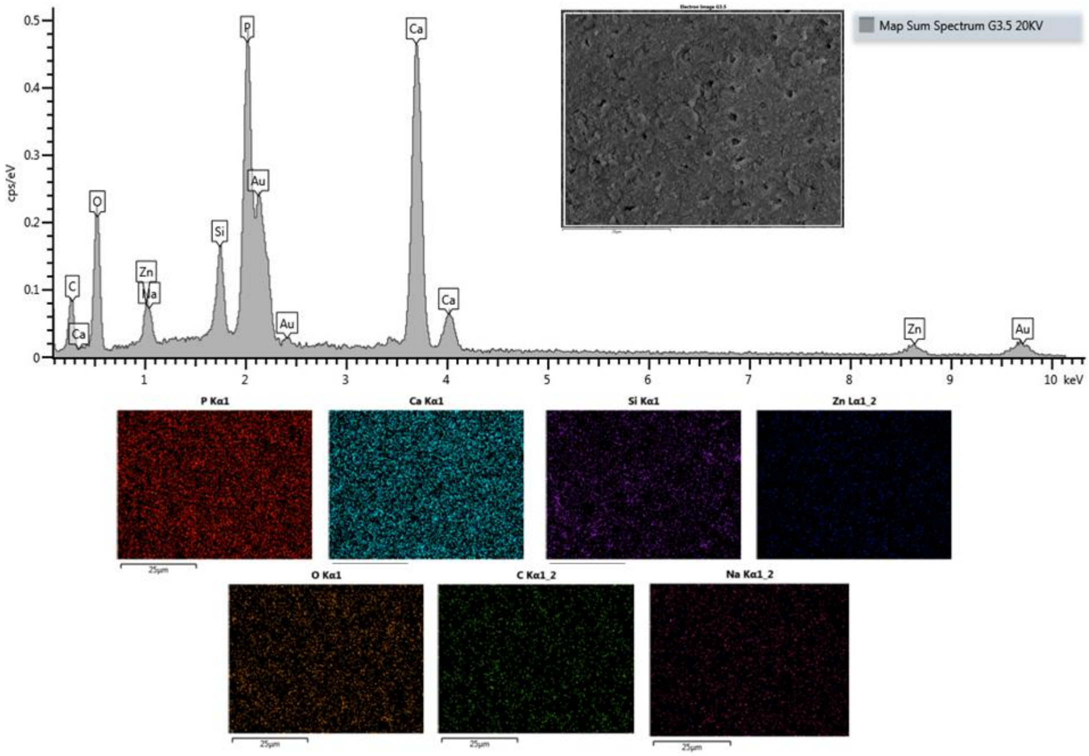


Figure 14: Energy dispersive X-ray spectroscopy (EDX) spectra from a representative sample of group 3 showing a high level of the phosphorus and calcium elements.

Figure 15 A and B shows low and high magnification secondary electron SEM images of the surfaces treated with the CSPS-based dentifrice. After fourteen days of treatment dentin surfaces appeared most completely covered by an inhomogeneous layer with a few or no open tubules discernible. From cross-sectional view it could be noticed a significant reduction or even looseness of tubule lumens and precipitates occluding them (figure 16). EDX chemical map taken alongside a secondary electron image of a mapped area showed that this occlusion deposits are predominantly composed of calcium and phosphorous. Additionally, discrete signs of titanium and sodium were also found (figure 17 and table 3).

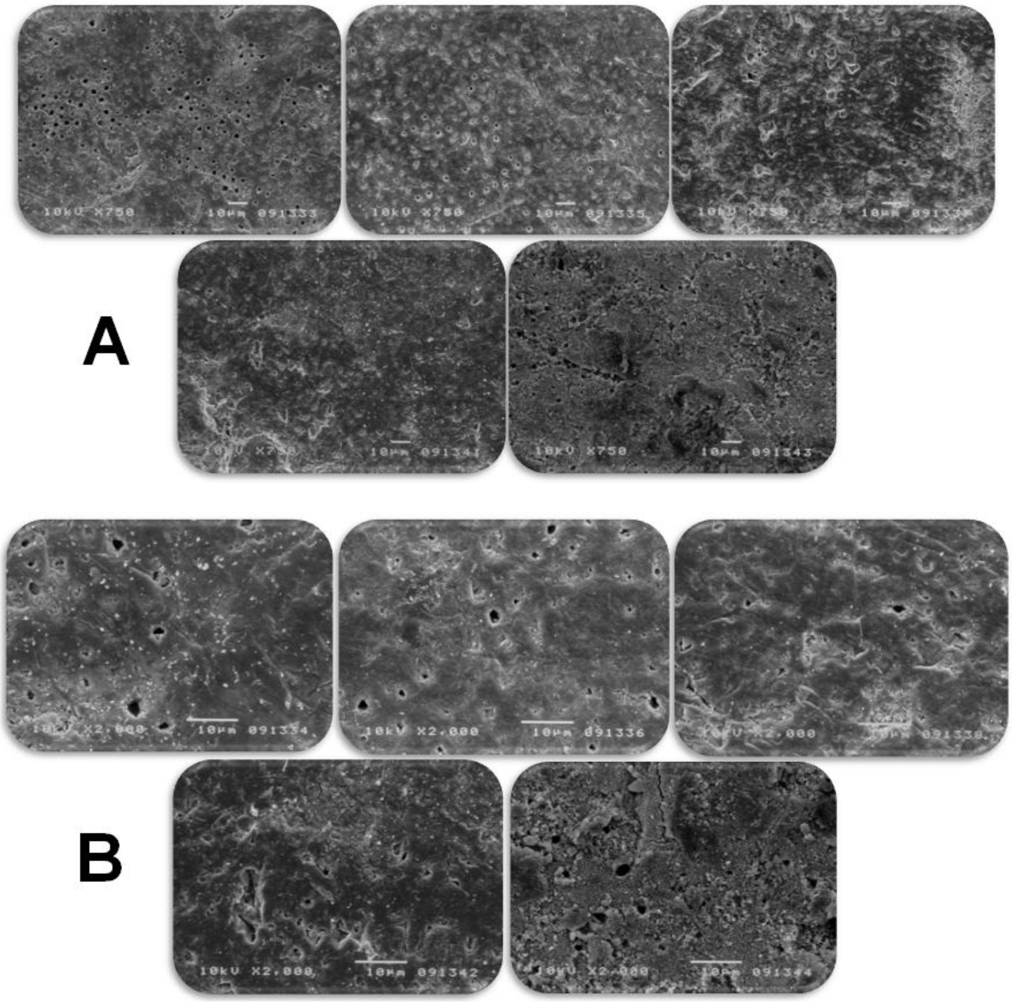


Figure 15: Representative photomicrographs of surface dentin samples brushed with Sensodyne® Repair&Protect™ (group 4) after fourteen days treatment at a 750-fold (A) and 2000-fold (B) magnification. On the surface it can be observed an inhomogeneous covering layer with a few or no open tubules discernible.

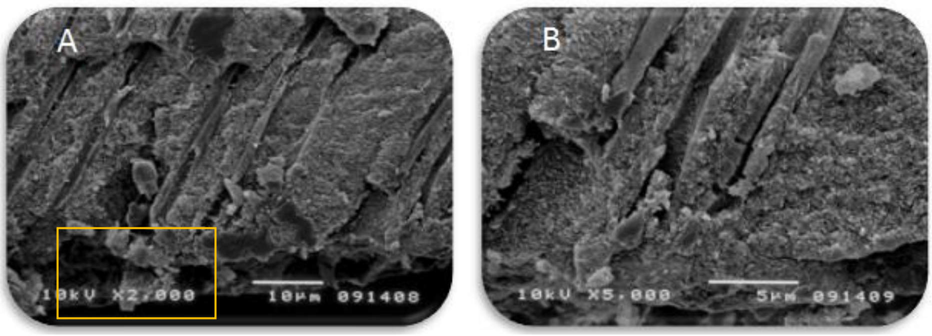


Figure 16: Representative photomicrographs of cross-sectioned dentin samples brushed with Sensodyne® Repair&Protect™ (group 4) after fourteen days at a 2000-fold (A) and 5000-fold (B) magnification. Significant reduction or even looseness of tubule lumens are evident with precipitates covering the surface.

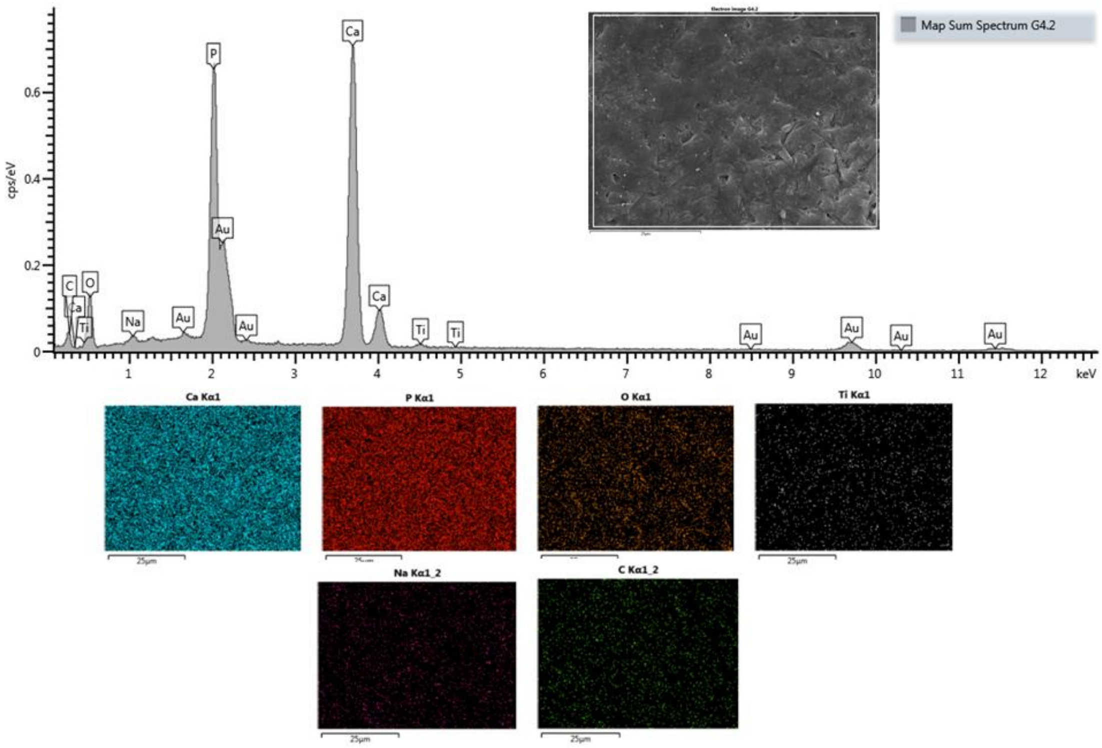


Figure 17: Energy dispersive X-ray spectroscopy (EDX) spectra from a representative sample of group 4 showing a high level of the phosphorus and calcium elements.

Discussion

Dentin hypersensitivity is considered one of the most prevalent painful condition of the oral cavity, but is poorly understood. An ideal dentin hypersensitivity treatment should mimic the natural desensitizing process, inducing changes in dentin that lead to rapid and lasting occlusion of dentinal tubules while being easy to apply and producing no side effects.[25] Nevertheless, such technology has not been yet reached and no gold standard treatment has been advocated justifying the interest of the conducted in vitro study.[26,27]

The dentine disc model has been used in several earlier studies and was considered to represent a close approximation of the *in vivo* situation.[28-30] As it is expected the treatment effects on different dentin substrates may not be exactly the same as several features can interfere on their response, such as tooth age; presence or absence of smear layer; differential density, diameter, direction and orientation of the dentinal tubules; presence or absence of highly mineralized peritubular dentine and variation of the intricate branching tubules system as well as the type and location of these branches.[31-33] In the attempt of trying to improve this model and obtain more reliable results all discs obtained from each tooth were sectioned in four quarters, which has permitted to homogenize the substrate across the different tested groups within the same tooth.

For over 30 years, a large spectrum of different toothpastes containing the most widely and recognized agents for dentinal hypersensitivity treatment has been used.[34] Their principal mode of action relies on the development of a smear layer both covering the dentine surface and occluding the dentin tubules, which is implicated in the reduction of the functional opening of the tubules inducing a reduction of the fluid flow and attenuation of the dentin hydraulic conductance.[35] In the present study we examined the surface and tubule-occluding properties of two recently introduced different active ingredients, a stabilized stannous fluoride (SFSH) and calcium sodium phosphosilicate-based (CSPS) toothpaste. The conclusions from our study support the findings of a well-developed occluding ability for both toothpastes encouraging their future use in clinical dentinal hypersensitivity trials. Therefore, we couldn`t reject our null hypothesis.

SEM analyses were performed using 750-fold and 2000-fold magnifications and were found to be suitable to evaluate dentin tubules morphology and occlusion as reported in other several studies.[36-39] Nevertheless, the 750-fold magnification should be considered a more representative view of the treated samples in that a relatively broad image is obtained from the surface where a considerable number of dentin tubules are identified. The 2000-fold magnification allowed a more precise visualization of dentin tubules, but the micrograph can include a more or less obliterated area of treated surfaces, which can accounts for discrete alterations of scores between these magnifications for the same evaluated surface. The statistically significant difference found between G1-G2 and G3-G4 at the 2000-fold magnification may be explained by the smaller sample size view. Nevertheless, the p values found for both difference between these groups is near to the interval confidence of 95% (p<0,05). Thus, the use of a 2000-fold magnification could be considered less reliable as the 750-fold for precise evaluation because limits the observational area.

According to our study both toothpastes were effective in dentin tubules occlusion, confirming other *in vitro* studies reports that have shown the effectiveness of both occluding technologies and most importantly, a sustainable stable surface occlusion against acid challenge or mechanical,[40-45] corroborating our findings. Nevertheless, in the present study both examiners reported in their evaluation that the occlusion pattern of dentin tubules for all samples was higher and more homogeneous for the CSPS-based dentifrice.

As expected, micrographs of open tubules were visualized from all samples of group 1 that were only subjected to a demineralization agent. Although in group 1 it was used the same artificial saliva as in group 2, the samples were not subjected to a brushing process, thus not creating smear layer on the surface. Chemical analysis (EDX) of group 1 showed high levels of carbon, oxygen and nitrogen and lower levels of calcium and phosphorous which correlates well with a demineralize surface where the collagen matrix is exposed.

Group 2, in which samples were brushed with artificial saliva twice daily for 14 days showed a considerable number of tubules obliterated. For these specimens, dentinal tubule occlusion can be attributed to calcium phosphate precipitation on the dentin surface, because artificial saliva is supersaturated with respect to hydroxyapatite.[46] Published studies showed that in some instances natural occlusion of tubules occurs because of the formation of smear layer or oral deposits of calcium phosphate derived mainly from the saliva.[47] However, these occlusions may be easily modified by, for example, tooth brushing or acid challenge, dislodging the tubule obstruction. This explanation supports the fact that the nature of the dentin hypersensitivity condition is such that frequent episodes of acute pain are followed by periods of quiescence.[47]

Concerning the dentifrice based on the stannous fluoride sodium hexametaphosphate formula, few in vitro studies were published until now, which makes it difficult to compare our data. Nevertheless, the present results have shown high effectiveness in dentinal tubules obliteration and surface coating after a twice-daily fourteen days treatment. Miller *et al*[22] found that when stannous fluoride anhydrous preparation is brushed onto dentine in vitro, a nearly complete coverage of the dentine surface and occlusion of tubules by a tin-rich surface deposit was observed. This finding indicates that the observed clinical efficacy of this preparation at relieving hypersensitivity is probably due to occlusion of tubules by a mixture of low solubility complexes of tin. According the EDX spectra obtained in our study, high contents of calcium and phosphorus was found at the surface besides lower levels of silicon (Si), zinc (Zn) and sodium (Na). The Si and Zn signals should have resulted from SiO_2 and ZnO_2 formation, additional ingredients of the toothpaste. Tubule occluding particles have been reported to be silica in specimens brushed with silica-containing dentifrices.[46] Therefore, if a dentifrice contains silica, occluding effects can also be attributed to its presence.[46] Surprisingly, tin element was not identified in our obtained spectra, although we could detect a discrete peak at the typical tin-specific L_α line at 3.443 keV.[48] Similar results were obtained by Ganss *et al*[49] whose work revealed that the amount of tin retained in sound dentin and on surfaces where the organic matrix was preserved was much lower than on dentin surfaces that underwent severe erosive conditions. Besides, they reported a considerably thick continuous layer covering sound dentin surface consisting mainly on Ca and P and relatively small amounts of tin emphasizing that the mechanism behind this covering is unclear, this is, how tin is complexed, precipitated or bounded on dentin tissue structure is currently not well known. Although in the present study the treated substrate was demineralized, the high calcium and phosphorus peaks in the spectra may indicate that organic matrix has been only lightly demineralized. Therefore, no accurate conclusions can be drawn on the source of the signal of Ca, P and O, as those elements are common in hydroxyapatite and, consequently on dentin.[49] Possibly, tin could be found more deeply in dentin, as it seems that tin uptake can be a diffusion control process as it may diffuse through the collagen structure in depth. Stannous fluoride chemical interaction can occur either with the mineral content by the formation of tin salts and/or with collagen or other dentin proteins that contains negatively charged groups capable of binding cations with strong affinity.[49] Therefore, the reduction and occlusion of tubule lumens that became evident after brushing treatment with this toothpaste could be in part due to both pathways. Additionally, some studies have reported about its good resistance against acid challenge.[41,44,45,49]

Despite the multi-functionality of this SFSH-based dentifrice, several *in vivo* studies published indicate that in the long-term, it has a significant effect in reducing sensitivity.[50-53] Schiff T *et al*[51] reported on an 8-week clinical trial where the subjects brushed twice daily and were evaluated for the air sensitivity (Schiff Air Index) and tactile sensitivity (Yeaple Probe Index) at 4 weeks and at the end of the trial. Oral-B & Pro-Expert® exhibited a statistically significant reduction in dentinal hypersensitivity for both evaluated index when compared to the negative control dentifrice at both weeks 4 and 8, thus providing evidence that the 0.454% stabilized stannous fluoride in this new dentifrice is effective in reducing the pain associated with hypersensitivity.

Another study reported by Blong *et al*[54] using a precise thermo-electric stimulator, demonstrated that a 0.4% SNF_2 gel was an effective agent in reducing dentinal hypersensitivity when used twice a day over a two week period. More recently, Thrash *et a*[52]*l* supported the theory the time required for a decrease in sensitivity is between two and four weeks from initiation of treatment. Thrash *et al*[52] compared a 0.4% stannous fluoride gel with an aqueous 0.717% fluoride solution and a placebo at 2, 4, 8, and 16-week intervals following a twice-daily application. The results indicated that subjects who applied the 0.4% SNF_2 reported significantly less sensitivity during the four to eight week period. Besides, the effect continued throughout the 16-week assessment period

The use of calcium sodium phosphosilicate in toothpaste is a relatively new approach in the treatment of dentin hypersensitivity however there are already a significant quantity of experimental and clinical data that demonstrates the efficacy of this compound as a desensitizing treatment agent, and also some in vitro data supporting its potential as a remineralization agent.[20,41,55-57] With regard to the quantitative score SEM evaluation and chemical analyses for this toothpaste containing CSPS, our results were similar to those reported by others authors.[40,41,43] Dentin samples treated surfaces showed a larger number of tubule obliteration, as well as a compacted protective mineralized surface layer proven by the obtained EDX surface map taken from inside the selected secondary electron image window where the main elements present were calcium and phosphorus and low levels of titanium (Ti) and sodium (Na). The Ti signal is thought to result from TiO_2 formation, an additional ingredient of the toothpaste.[40] It would also be expected to observe the silicon (Si) signal due to its presence as an ingredient of the toothpaste as mentioned by Earl *et al.*[40] Nevertheless, this element was not detected in the present study, possibly because the silica rich layer is far behind the surface deposition hydroxyapatite like surface layer after developed after the fourteen days treatment protocol. Perhaps it would be measurable if we have taken an EDX area or point measurement spectrum from the cross-sectional SEM image along the occluded tubule. This formed surface layer was previously described to be a mixture of nano-crystalline, micro-crystalline and amorphous material composed by an hydroxyapatite-like material resulting in the formation of a calcium-phosphate enriched layer resistant to acid and mechanical challenges.[40-42] This stabilized surface layer can be attributed not only by the physically occluded tubules and surface but also due to the potential chemical interaction of CSPS with exposed type I collagen fibers enabled by the negative surface charge induced as soon as those particles initiate its reactivity with the substrate.[58,59] These findings are consistent with clinical findings reported in the literature indicating CSPS to be an effective agent for reducing tooth sensitivity assessed by randomized controlled clinical trials.[55,56,60-62]

Salian *et al*[62] compared a commercially available toothpaste containing 5% CSPS to a positive control toothpaste containing 5% potassium nitrate and a negative control toothpaste without desensitizing ingredients in a total of thirty subjects, and follow-up assessments using standardized application of tactile, air and ice challenge were made after two and four weeks of product use. All sensitivity scores in the group using the toothpaste containing CSPS were significantly better than the positive and negative control groups. Pradeep and Sharma[56] also conducted a six-week study in India comparing a commercially available toothpaste containing 5% CSPS to one containing 5% potassium nitrate and to a negative control toothpaste using air and cold water as stimuli in a total of 110 subjects with follow-up after two and six weeks. Comparatively, air and water sensitivity scores for the CSPS group were significantly better than the positive and the negative control groups at both time points tested.

Conclusions

According to the present in vitro study no statistically significant differences on the occlusion ability of dentin tubules were found between both tested toothpastes. However, the qualitative analysis made by both operators indicated a more homogenous dentin occlusion achieved with Sensodyne® Repair&Protect™.

From the chemical point of view brushing either with Oral-B® Pro Expert or Sensodyne® Repair&Protect™ conduces mainly to similar phosphorus and calcium deposition over dentin tubules.

Brushing with artificial saliva produces a similar dentin tubules occlusion when compared to brushing with Oral-B® Pro Expert.

Future perspectives

This *in vitro* study results showed that both toothpastes application resulted in good dentin tubules obliteration scores. However, further studies should be performed with higher sample number using lower magnifications for wide area evaluation, while subjecting samples to daily erosive or mechanical challenges to evaluate the stability of the present results.

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