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Original article

Investigation on the Mode of Action of Three Desensitizing Agents Using Scanning Electron Microscopy and Spectroscopy: An In-Vitro Study

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ABSTRACT:

Aim: This *in-vitro* study aimed to ascertain the mode of action of three desensitizing agents namely, Arginine-containing, Propolis-containing and Potassium Nitrate-containing dentifrices using scanning electron microscopy and energy-dispersive X-ray spectroscopy.

Materials and methods: Ninety dentin slices from non carious human premolar teeth were used for the study. The teeth were randomly divided into three groups (n=30), according to the type of dentifrice applied: Group I: Arginine-containing paste, Group II: Propolis-containing paste and Group III: Potassium nitrate –containing paste. Each group was divided into two subgroups (n=15) for the assessment by two different techniques: Subgroup A: Scanning electron microscopy examination and Subgroup B: Energy-dispersive X-ray spectroscopy examination. SEM was used to assess the tubule occlusion and the change in the diameter of the dentinal tubules. EDXS was used to characterize the elemental composition of the occlusive material. Analysis of agents, both prior to and after application on dentine discs was performed for comparative purposes. Statistical analysis was done using one-way analysis of variance (ANOVA) and the Bonferroni post hoc test for multiple comparison.

Results: The dentin discs treated with arginine-containing paste (93.220% by SEM) showed statistically ($p<.001$) highest mean percentage of occluded tubules followed by Potassium Nitrate–containing paste (34.777% by SEM) and Propolis-containing paste (13.580% by SEM). There was a strong evidence ($p<.001$) that group I(68%) was most effective in reducing the mean tubule diameter, while group II(47%) and III(44%) did not produce a significant reduction. The EDXS studies show that the dentin surface deposit and occluded tubule plugs contain high levels of calcium, phosphorus, fluorine, silica in group I and III treated specimens.

Conclusion: The study presented shows successful dual action properties- tubule occlusion and nerve depolarization of arginine-containing paste and potassium nitrate-containing paste and partial tubule occluding property of propolis-containing paste as evaluated by scanning electron microscopy and energy-dispersive X-ray spectroscopy.

KEYWORDS: Desensitizing agents, Scanning electron microscopy, Confocal laser scanning microscopy, Energy-dispersive X-ray spectroscopy, Dentin hypersensitivity.

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INTRODUCTION

The problem of dentine hypersensitivity is an enigma that has interested many clinicians and is characterized by 'pain derived from exposed dentine in response to chemical, thermal, tactile or osmotic stimuli which cannot be explained as arising from any other dental defect or pathology[1].

Applying Brannstrom's concept of hydrodynamic theory, desensitizing agents have been developed to occlude the dentinal tubules which reduce the functional radius of dentinal tubules thereby reducing the permeability and dentinal sensitivity[2]. Currently, there are two main approaches for the treatment of root dentin sensitivity–tubule occlusion and blocking nerve activity through direct ionic diffusion[3,4].

Of the various methods and materials for the relief of dentinal sensitivity, dentifrices are widely indicated, particularly because of their low cost, ease of use and home application[1]. Dentifrices containing potassium, sodium fluoride, oxalates may take up to 2-4 weeks to relieve symptoms. The advent of over-the-counter desensitizing toothpaste containing breakthrough pro-argin technology takes less than two weeks to demonstrate faster degree of success in reducing hypersensitivity[3].

To validate a desensitizing material for its tubule occlusion, morphology, depth of tubule occlusion and identify the elemental composition various techniques have been used in the literature. SEM and EDXS are the powerful techniques to assess these parameters[5,6]. Very few studies have been performed with a combination of techniques to assess dentinal hypersensitivity. The purpose of this *in-vitro* study is to compare the mode of action of three desensitizing agents using scanning electron microscopy and energy dispersive x-ray spectroscopy.

MATERIALS AND METHODS

Three commercially available toothpastes were investigated: Product 1, Colgate Sensitive ProRelief (Colgate-Palmolive Company); Product 2, Propolis (Manuka Health New Zealand; Product 3, Colgate Sensitive (Colgate-Palmolive Company).

SPECIMEN PREPARATION

Ninenty dentin slices, approximately 800µm-thick, were cut from the crown section of human premolars in a parallel manner, slightly below the enamel-dentin junction, using slow speed diamond disc (DFS, Germany) attached to a micromotor straight hand piece (NSK, Japan) with copious water irrigation. The dentin slices were then polished on one side using 600 grit wet paper to create an even and uniform surface. The side that was polished magnified printed text when the specimen were placed over it. Each specimen was polished for approximately 30 seconds to make each specimen shiny. The polished specimens were then placed in a jar of deionized water and sonicated for 10 minutes to remove the abrasive. After sonication, the specimens were rinsed with water. The tubules were opened by etching the dentin specimens in a Petri dish with a 1% citric acid solution, using mild agitation for 20 seconds. After etching, the specimens were rinsed with deionized water and finally sonicated once again for 10 minutes. The etched and sonicated specimens were stored in a phosphate buffer saline (PBS, pH= 7).

EXPERIMENTAL GROUPS AND TREATMENTS

The specimens were divided into 3 groups of equal size (n=30) for quantitative and qualitative evaluation of arginine-containing, propolis-containing and potassium nitrate- containing pastes.

Group I: To detect the mode of action after application of Arginine-containing paste; Group II: To detect the mode of action after application of Propolis-containing paste; Group III: To detect the mode of action after application of Potassium Nitrate–containing paste.

Each group was divided into two subgroups for the assessment of mechanism of action by two different techniques. Subgroup A (15 teeth) - Scanning electron microscopy examination; Subgroup B (15 teeth)- Energy-dispersive X-ray spectroscopic examination.

ASSESSMENT OF DENTIN SPECIMENS PRIOR TO APPLICATION OF TEST PRODUCT USING SEM AND EDXS

Forty five dentin discs, 15 from each group were examined by low energy SEM (Hitachi SU6600, Tokyo, Japan), to ensure that the dentin tubules were in an open unoccluded state. Photomicrographs of representative dentin areas were taken at magnification of 1000x and 5000x. This ensured that the specimens were of sufficient quality to be used for further experiments.

On each Scanning electron micrograph, five random tubule diameters (µm) were measured and the average of the diameters was reported. The

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NIH Image software was used to measure the diameter of the dentinal tubules. The values were charted and recorded.

45 dentin specimens, 15 specimens from each group - group I,II and III were examined by Energy-dispersive X-ray spectroscope. The spectrometer attached to the SEM (Hitachi SU6600, Tokyo, Japan) was used to determine the chemical elements in the untreated discs. The spectrum was obtained at 15kV. This provided atomic percent of the presence of Carbon (C), Oxygen (O), Nitrogen (N), Calcium (Ca), Phosphorus (P), Sodium (Na), Fluorine (F), Silicon (Si) and Potassium (K).

APPLICATION OF TEST PRODUCTS

Test product was added onto the dentin discs of all the 90 specimens. Dentin discs were placed on a microscope slide with the polished side up. The sample were wetted with PBS buffer, and then test product was applied to the dentin surface, mixed with the PBS and spread across the entire surface using gentle strokes and a camel hair brush. The desensitizing material, the test product was added to dentin specimens. For specimens in group I (30 Teeth - Arginine-containing paste was applied, Group II (30 Teeth)-Propolis-containing paste and for specimens in Group III (30 Teeth) - Potassium Nitrate–containing paste was applied.

The samples were left undisturbed for 15 minutes at room temperature. After this, the samples were placed in a jar containing 30 ml of PBS buffer, where they remain for 15 minutes while stirring. The samples were then gently rinsed to ensure removal of any excess product from the surface. This treatment was repeated five times and done in duplicate. The samples were dried prior to analysis.

ASSESSMENT OF DENTIN SPECIMENS AFTER THE APPLICATION OF TEST PRODUCT

Following treatment with desensitizing agents, the discs were mounted on SEM stubs and sputter coated with a 15nm layer of gold (Hitachi E-1010, Tokyo, Japan) to aid conductivity. SEM examination was done at an operating voltage of 15kV. Each specimen was considered as a sample unit, and scanning electron micrographs was obtained from the center of each sample at 1000x, 5000x magnification for further detailed analysis. The fully and partially occluded tubules in each micrograph were marked using a computer program (Adobe Photoshop, Adobe). The total number of tubules was also counted in each field. All the micrographs calculated were at magnification of 1000x. The percentage of partially and/or fully occluded tubules was calculated for each representative micrograph using the following formula.

$$\text{Percentage of partially or fully occluded tubules} = \frac{\text{Number of partially/totally occluded tubules}}{\text{Total number of tubules}} \times 100$$

On each Scanning electron micrograph, five random tubule diameters (µm) were measured and the average of the diameters was reported. The NIH Image software was used to measure the diameter of the dentinal tubules. The measurements were recorded for discs treated with group I, II and III pastes. The percentage reduction in the diameter of the dentinal tubules was calculated for each group by:

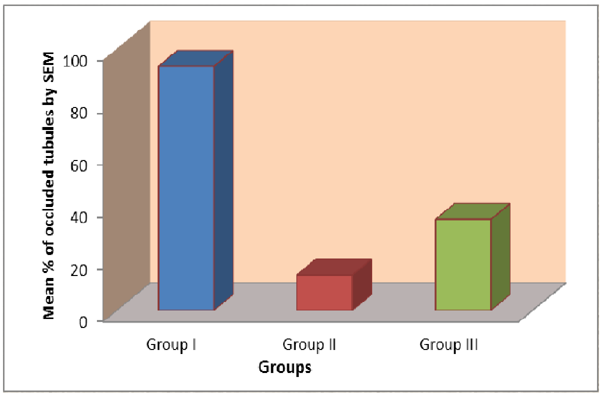
$$\text{Percentage of partially or fully occluded tubules} = \frac{\text{Number of partially/totally occluded tubules}}{\text{Total number of tubules}} \times 100$$

Post-treatment spectroscopic values of the elements were charted.

RESULTS

SCANNING ELECTRON MICROSCOPY

Group IA (Arginine-containing paste) showed the highest mean percentage of tubule occlusion (93.220%) followed by group IIIA (potassium nitrate- containing) paste (34.777%) and group IIA (propolis-containing) paste (13.580%). Very high significant difference was observed between the groups as evaluated by SEM (*p<001*). Intergroup comparison shows high significant difference between all groups when evaluated by SEM (*p<.001*).(Graph 1)



Graph 1. Bar graph showing the mean percentage of occluded tubules of Group I, Group II and Group III as observed under the SEM.

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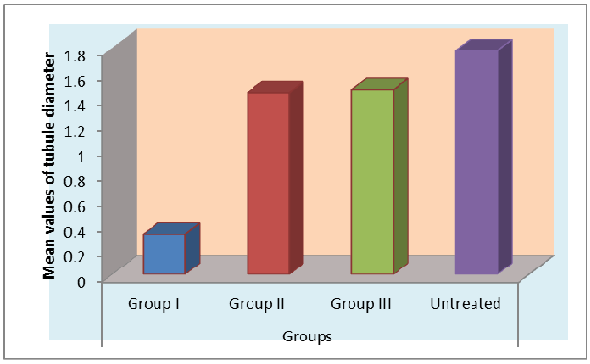
DENTINAL TUBULE DIAMETER

Tubule diameter reduced after application of Group I (68%), II(47%) and III(44%) pastes compared to the diameter of untreated samples. A high statistically significant difference was observed for Group I samples (*p<.001*) whereas, no significant variation was seen for Group II and III samples.(Graph 2).



Graph 2.Bar graph showing the mean values of tubule diameters (µm) observed by the SEM.

ANOVA, F=27.697 *p<0.001* HS.

ENERGY-DISPERSIVE X-RAY SPECTROSCOPY

Carbon (59.168%), Oxygen (25.456%), Nitrogen (11.553%), Calcium (2.392%), Phosphorus (1.4953%) and Sodium (0.02%) were detected in the untreated samples using EDXS. Fluorine, Silicon and Potassium were not found in the untreated samples using EDXS. EDXS detected the presence of Carbon (45.307%), Oxygen (27.927%), Nitrogen (11.412%), Calcium (6.2966%), Phosphorus (4.1573%), Sodium (2.2306%), Fluorine (1.425%), Silicon (1.406%) and Potassium (0.301%) after application of arginine-containing paste (Group I). Calcium, Phosphorus, Sodium, Fluorine and Potassium levels significantly increased. EDXS detected the presence of Carbon (48.366%), Oxygen (27.413%), Nitrogen (14.362%), Calcium (3.2606%), Phosphorus (2.5366%), Sodium (1.532%) and Silicon (2.528%) after application of propolis-containing paste (Group II). EDXS detected the presence of Carbon (47.103%), Oxygen (26.889%), Nitrogen (16.346%), Calcium (2.0626%), Phosphorus (2.0746%), Sodium (1.4486%), Fluorine (0.7386%), Silicon (1.7513%) and Potassium (0.9306%) after application of potassium nitrate-containing paste (Group III). Calcium, potassium, silica and fluorine levels showed a significant variation. (Table 1)

Table 1. EDXS analysis of dentin samples before and treatment.

Sample	ATOMIC PERCENT (%)								
	C	O	N	Ca	P	Na	F	Si	K
Untreated	59.168	25.456	11.553	2.392	1.4953	0.02	-	-	-
	After application of desensitising pastes								
Group I	45.307	27.927	11.412	6.2966	4.1573	2.2306	1.425	1.406	0.301
Group II	48.366	27.413	14.362	3.2606	2.5366	1.532	-	2.528	-
Group III	47.103	26.889	16.346	2.0626	2.0746	1.4486	0.7386	1.7513	0.9306

C= Carbon, O=Oxygen, N=Nitrogen, Ca=Calcium, P=Phosphorus, Na=Sodium, F=Fluorine, Si=Silicon, K=Potassium.

DISCUSSION

A possible approach to reducing or eliminating the painful symptoms of dentin hypersensitivity is by reducing the fluid movement through the narrowing or occlusion of tubule openings[7]. The need for a fast, lasting relief from dentin sensitivity pain by the modern consumers resulted in the introduction of the pro-argin technology[8]. The incorporation of arginine into dentifrices was originally reported in the late 1990s.[9] Propolis is a natural, non-toxic resin produced by honey bees that has been used for years[10]. The vast majority of desensitizing toothpastes, representing approximately 10% of the global toothpaste market, contain a potassium salt to "numb" the pain of dentin hypersensitivity using various concentrations[11]. Potassium salts have been shown to interrupt the neural response to pain stimuli[12].

Hence the present study compared the mode of action of arginine-containing, propolis–containing and potassium nitrate-containing pastes to treat dentin hypersensitivity by SEM and EDXS.

In the present study, the dentine disc model was employed to examine the tubule-occluding properties of these agents and which has found to be a useful screening method by Pashley, Greenhill, Absi, Addy & Adams. All the dentin samples in this study were etched with 1% citric acid to maintain wide, patent dentinal tubules and thereby simulate the open, tubules of sensitive dentin[13].

All the tested dentifrices showed the ability to occlude the dentinal tubules as demonstrated by the SEM results which proves that they act by blocking the dentinal tubules. Arginine-containing tooth paste (group I) exhibited maximum occluding capability (93.220%) which was twice that of potassium nitrate-containing paste (group III) and five times than propolis-containing paste (group II). Similar data was obtained by Lavender et al. and Petrou et al. showing almost complete obliteration of the dentinal tubules when studied by SEM and CLSM[3,4]. Kleinberg in earlier research studies has proposed the mechanism of action of arginine-containing paste. The combination of arginine and calcium carbonate forms a plug that occludes the dentin tubules[3,4].

Potassium nitrate-containing paste (group III) exhibited a tubule-occluding ability twice that of propolis-containing paste (group II). Our results are in agreement with the *in vitro* studies of Wang et al., Paes Leme et al. and Knight et al. who have tested the occlusion efficacy of Potassium nitrate-containing paste[2,14]. The higher clinical efficacy of Potassium nitrate-containing tooth paste can be explained by the dual action of these pastes[15].

The present study along with the study by Sales-Peres[10] has revealed that dentinal tubules were only partially occluded after treatment with propolis. For the first time propolis has been tested in the form of a commercial toothpaste in treating hypersensitivity and there is insufficient data with regard to both its potential mode of action and its clinical efficacy. A positive clinical assessment however, was reported by Mahmoud et al. (1999) and Madhavan et al. (2012) using propolis in treating hypersensitivity where propolis was lab formulated[16].

After treatment with the arginine-containing paste (group I), EDXS analysis of the dentin surface showed that the levels of calcium and phosphorus increased with a decrease in carbon content. These results provide evidence that the treated surface had remineralized, and was covered by a dentin-like mineral of calcium and phosphate. For the first time potassium ion (0.3%) was detected in arginine-containing paste. Markowitz et al. proposed that the desensitizing effects of potassium ions were due to increased potassium ion concentration ($[K^+]$) in the extracellular fluids surrounding the intradental nerves, which gives an insight to its mode of action *via* nerve depolarization[17].

In the present study there was a rise in the atomic percentage of calcium, phosphorus and silicon which was due to the silica and calcium containing abrasives in the paste formulation. The studies of Prati and co-workers (2002) have established that dentifrice abrasives have an occluding effect on dentinal tubules, thereby justifying the SEM and CLSM observations of the present study[8]. Propolis-containing paste (group II) was the only paste in the present study not to contain a source of fluoride ions which justifies its absence in the spectroscopic analysis.

Although potassium nitrate-containing dentifrices were designed to deliver potassium ions (0.93%) to reduce nerve excitability in hypersensitive teeth, our study in corroboration with studies of Wang et al.[14] showed that these toothpastes had the ability of occluding dentinal tubules. This effect was possibly due to the silica and calcium containing abrasives rather than their clinical active ingredients.

In the present study, the mean diameter of dentinal tubules in group I, II and III showed a reduction of 68%, 47% and 44% respectively showing the efficiency of arginine-containing paste. However, this was an *in-vitro* study and therefore cannot be directly applied to clinical use of these desensitizing agents. Further clinical studies should be conducted to clarify the dual action of arginine-containing pastes.

CONCLUSION

It can be concluded that the three tested dentifrices produced increased dentinal occlusion, however Arginine-containing paste exhibited superior tubule occluding properties. For the first time potassium ions were detected in arginine-containing dentifrice-treated discs, which may confer an additional action of nerve depolarization for treating sensitivity.

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