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Effects of Dentifrices Containing Nanohydroxyapatite on Dentinal Tubule Occlusion—A Scanning Electron Microscopy and EDX Study

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Abstract: This in vitro study evaluated the effects of dentifrices containing nano-hydroxyapatite (n-HAp) on dentinal tubule occlusion and on mineral deposition. Dentin specimens of ten human teeth were submersed for 30 s in 40% citric acid and then randomly divided into four groups (three study groups and one control group). In the study groups, the dentin samples were exposed to three different n-HAp toothpastes: Karex (Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany), Biorepair Plus Sensitive (Coswell SpA, Bologna, Italy), and Dr. Wolff's Biorepair (Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany); in the control group no toothpaste was applied. All of the samples were evaluated using scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analysis. In the control group all of the samples showed a frank and wide opening of the dentinal tubules, whereas in the study groups different degrees of tubule closure by mineral depositions were observed. Toothpastes containing n-HAp determined a significant occlusion of dentinal tubules and a significant increase of mineral deposition on the dentin surface. All three tested toothpastes showed similar results regarding the degree of dentinal tubule closure. Varying degrees of differences in calcium, phosphate, carbon, and oxygen ion concentrations among the three tested toothpastes were obtained.

Keywords: hydroxyapatite; dentifrices; dentin; tubule closure; dentin hypersensitivity

1. Introduction

Dentin hypersensitivity (DH) was described in 2009 by the FDI World Dental Federation as a "short sharp pain arising from exposed dentin most commonly at the tooth cervical area in response to stimuli (typically thermal, evaporative, tactile, osmotic or chemical), but which cannot be ascribed to any other dental defects, diseases or restorative treatments" [1]. Increased global average life expectancy and increased number of teeth because the population is now aware of the benefits of prevention and the importance of good oral hygiene [2,3] led to increased prevalence of DH, which commonly can range up to 74% [4,5].

Different theories were proposed throughout the years to explain the pathogenesis of DH, the hydrodynamic theory of Brännström being widely accepted nowadays. This theory postulates that external stimuli can rapidly dislocate the contents of dental tubules, distorting the nerve at pulp or predentin level or damaging the odontoblast cells and ultimately causing pain [2,6–9]. This is the explanation for two treatment strategies used in practice: dentinal tubule occlusion and nerve excitation prevention [10]. The agents in the first category determine the formation of an intratubular precipitate or the formation of an artificial smear layer over the dentin that blocks the entry into the tubules. Deposition of the thin coating layer determines the occlusion of the tubules, but also remineralization of the exposed dentin surface [11]. However, none of the agents in this category have been considered as a gold standard for dentinal tubule occlusion [12]. Various products are available on the market to be used either by the patients at home or by the dentists in the office, but the most common treatment of DH is the use of dentifrices [13,14].

Recommended toothpastes for DH contain different active ingredients such as: fluoride, calcium oxalate, calcium carbonate–phosphate, strontium acetate, arginine, and hydroxyapatite (HAp) [13,15–17]. HAp is the major inorganic component of natural teeth and bone and previous studies showed that nano-sized particles have similarities in morphology and structure to the tooth enamel apatite crystals [18,19]. Some studies and reviews have mentioned the capacity of this biofunctional material to remineralize enamel and dentin [19–22]. Nano-hydroxyapatite (n-HAp) was considered a promising active ingredient used for the treatment of DH due to high biocompatibility and bioactivity [23–25]. In toothpastes, HAp was included in the form of nanocrystals because they dissolve easier in this form [19,20,26]. Crystals of n-HAp included in dental products have a dimension of 50–1000 nm, which enables them to act like fillers [27]. These products can penetrate and block the exposed dentinal tubules which are responsible for DH. There is no common opinion in the literature regarding the efficiency of n-HAp based products used in DH treatment. Some studies have demonstrated higher desensitizing action and dentin remineralizing potential of n-HAp desensitizing agents [28,29], while other clinical studies have shown an equivalent result of n-HAp to other desensitizing agents [30].

This study aims to compare the effectiveness of three different commercial n-HAp toothpastes on dentinal tubule occlusion and on dentin mineral deposition by scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analysis. The null hypothesis was that there is no significant difference regarding tubule occlusion between the three n-HAp dentifrices.

2. Materials and Methods

2.1. Sample Preparation

Ten extracted human permanent third molars were collected anonymously in the Oral Surgery Department/School of Dentistry at the University of Medicine and Pharmacy “Grigore T. Popa” Iași. A written informed consent form of biological materials used during the study was voluntarily obtained from the patients. The research was conducted in full accordance with the national research law 206/24.05.2004 and the Declaration of Helsinki, the protocol being approved by the Ethics Committee of the University (project no 3390). The teeth inclusion criteria were the presence of a complete crown, the absence of caries, cracks, or fillings. The teeth were cleaned to remove the soft tissues and stored in distilled water.

From each tooth, two dentin discs were obtained from mid-coronal part by cutting the tooth in two sections perpendicular to the long axis with a diamond disc (NTI-Kahla GmbH, Germany) at low speed under cooling water. Each dentin disc with a thickness of 1 mm was then cut in two half-disks in order to obtain four dentin pieces from each tooth. All the dentin samples were wet polished using 600-grit SiC papers. To open the dentinal tubules and to simulate the sensitive tooth model, all of the dentin half-discs were submersed for 30 s in 40% citric acid (Cerkamed, Poland). Then they were rinsed thoroughly with distilled water, followed by ultrasonic bathing for 10 min. After that the specimens were randomly and equally assigned to four groups. In groups 1–3, considered as study groups, three commercial n-HAp desensitizing toothpastes were applied on dentin disks and in group 4, considered as the control group, no toothpaste was applied. The toothpastes selected for the study groups were: Karex (Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany)—applied in group 1, Biorepair Plus Sensitive (Coswell SpA, Bologna, Italy)—applied in group 2, and Dr. Wolff’s Biorepair

(Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany)—applied in group 3. Details regarding the chemical ingredients of the toothpastes are presented in Table 1.

Table 1. Chemical ingredients of the desensitizing toothpastes.

KAREX	Hydroxyapatite, Xylitol, Hydrated Silica, Tetrapotassium Pyrophosphate, Zinc Chloride, Cetylpyridinium Chloride, Sodium Methyl Cocoyl Taurate, Sodium Cocoyl Glycinate, Aqua, Glycerin, Phosphoric Acid, Silica, Hydrogenated Starch Hydrolysate, Cellulose Gum, Aroma
BIOREPAIR PLUS SENSITIVE COSWELL	Aqua, Zinc Hydroxyapatite, Glycerin, SorbitoI, Cellulose Gum, PEG-32, Silica, Sodium Myristoyl Sarcosinate, Sodium Methyl Cocoyl Taurate, Aroma, Sodium Saccharin, Citric Acid, Phenoxyethanol, Benzyl Alcohol, Sodium Benzoate
DR. WOLFF'S BIOREPAIR	Aqua, Zinc Hydroxyapatite, Hydrated Silica, Glycerin, Sorbitol, Silica, Aroma, Cellulose Gum, Sodium Myristoyl Sarcosinate, Sodium Methyl Cocoyl Taurate, Tetrapotassium Pyrophosphate, Zinc Pca, Sodium Saccharin, Phenoxyethanol, Benzyl Alcohol, Propylparaben, Methylparaben, Citric Acid, Sodium Benzoate.

The toothpastes were applied on the surface of the dentin disks using a brushing protocol which was described in some previous studies [31]. A tooth brushing machine designed to operate with back-and-forth movement at 1.5 Hz (250 g vertical load), with an amplitude of 30 mm (15 mm in each direction), and a frequency of 60 cycles/minute was used. The samples were brushed for 2 min, twice a day, for 14 days. The device was equipped with 4 stations to place the samples. Medium straight bristle toothbrushes (Classic Deep Clean, Colgate-Palmolive Company, New York, NY, USA) were placed in special attachments aligned parallel to the base. Abrasive slurries were prepared by mixing water and toothpastes (2:1 by volume). Between the tooth brushing sessions all the samples were stored in artificial saliva prepared according to AFNOR NF S90-701 standard procedure.

2.2. Scanning Electron Microscope (SEM) Analysis

Dentin samples were evaluated using scanning electron microscope VEGA II LSH (TESCAN, Brno, Czech Republic). Ten images at 2000× magnification were obtained of each dentin sample. The images were then assessed independently by two well-trained examiners, blinded to the tested toothpastes, to evaluate the degree of dentinal tubule occlusion on a five-grade scale, according to the tubule occlusion classification scoring system used in previous studies: 1 = occluded (100% of tubules occluded); 2 = mostly occluded (50–< 100% of tubules occluded); 3 = partially occluded (25–< 50% of tubules occluded); 4 = mostly unoccluded (<25% of tubules occluded); 5 = unoccluded (0%, no tubule occlusion) [26]. Each examiner counted the tubules in each of the 2000X images and established individually the dentinal tubule occlusion score on every image. If there was a disagreement in scoring between the two examiners, they re-examined the image until they arrived to a common opinion. For each sample, the average score of tubule occlusion from ten image evaluations was used for analysis.

2.3. Energy-Dispersive X-ray (EDX) Analysis

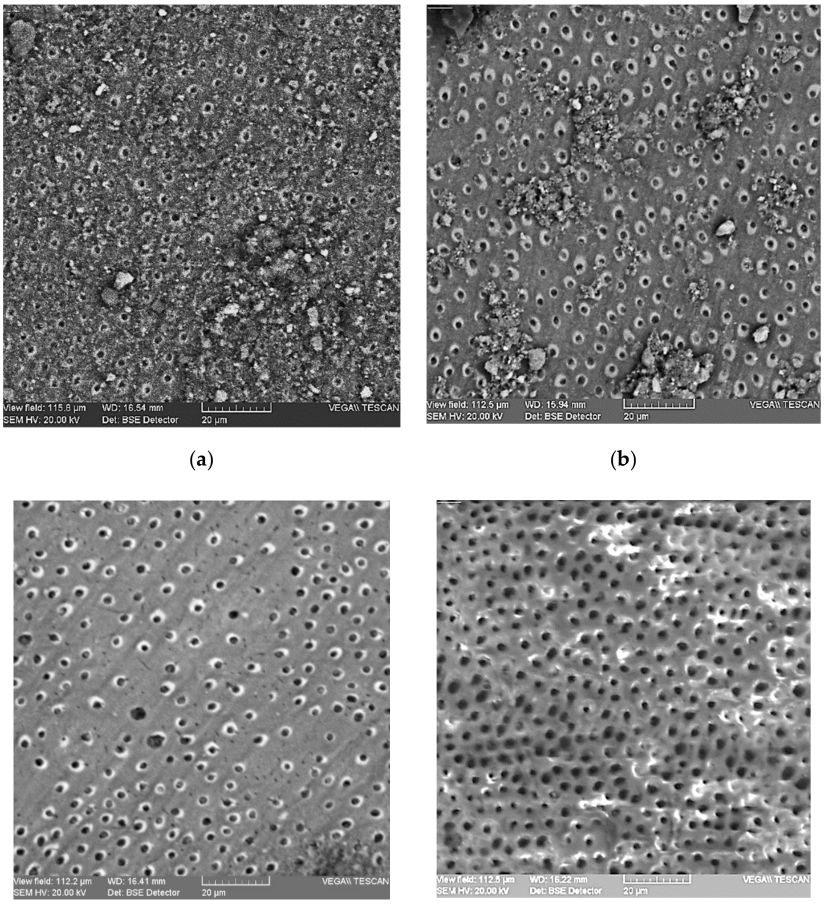
All of the specimens were examined by energy-dispersive X-ray microanalysis (QUANTAX QX2, BRUKER/ROENTEC, Berlin, Germany) for chemical determinations. Standard chemical composition determinations using PB-ZAF database on a selected area were used. Qualitative evaluation of the chemical elements revealed carbon (C), calcium (Ca), phosphorus (P), and oxygen (O) as dominant components. Quantitative evaluations of ion concentrations (wt%) were performed in ten different areas of each dentin sample. The ion concentrations on each sample were reported as the average value of ten determinations.

2.4. Statistical Analysis

The statistical software SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Inter-examiner agreement on dentin tubule occlusion scores evaluated on SEM images was established using the Kappa test. The Kolmogorov–Smirnov normality test and nonparametric Mann–Whitney test were used to compare the dentin tubule occlusion values in groups. The Levene homogeneity of variance statistical test, the Kruskal–Wallis nonparametric test, and post hoc LSD were used to compare the percentages of chemical elements in the control and study groups.

3. Results

SEM images of some samples in the control and study groups are presented in Figure 1. In the control group, all of the samples showed a frank and wide opening of the dentinal tubules (Figure 1d), which confirmed the sensitive tooth model. In the study groups, obvious closure of the tubules by mineral depositions was observed, with different percentages of tubule occlusion being registered on the same sample or in different samples from the same group (Figure 1a–c). While most of the samples presented all the tubules partially occluded (as presented in Figure 1a,b), other samples presented completely unoccluded tubules beside partially occluded tubules. Mineral deposition on the dentin surface was also observed mostly on the samples in groups 1 and 2 (Figure 1a–c).



(**a**) (**b**)

(**c**) (**d**)

Figure 1. SEM micrographs of dentin samples in study groups (**a**–**c**) and control group (**d**). (**a**) SEM image (2000×) of a dentin sample in group 1 (score 1). (**b**) SEM image (2000×) of a dentin sample in group 2 (score 1). (**c**) SEM image (2000×) of a dentin sample in group 3 (score 2). (**d**) SEM image (2000×) of a dentin sample in group 4 (score 5). SEM images on the dentin surface showing dentinal tubule occlusion with different scores in the study groups (group 1: Karex toothpaste, group 2: Biorepair Plus Sensitive toothpaste, and group 3: Dr. Wolff's Biorepair toothpaste) and in the control group (group 4).

Strong inter-examiner agreement on dentine tubule occlusion evaluated on SEM images was recorded (Kappa value of 0.961). The mean score values for dentin tubule occlusion and the number of samples specifically scored for tubule occlusion in each group are presented in Table 2. In group 3, the number of the samples with a score of 4 was higher than in groups 2 and 1. An equal number of samples with a score of 1 was recorded in groups 1 and 3 and an equal percentage of samples with a score of 3 was recorded in groups 2 and 3. The highest number of samples with a score of 2 was registered in group 1, followed by group 2 and 3, and the highest number of tubule closure with a score of 4 was obtained in group 3, followed by group 2 and group 1. Irrespective of the group, none of the samples in the study groups presented complete closure of dentinal tubules.

Table 2. The number of samples in the control and study groups scored with values between 1–5 for tubule occlusion.

	Number of Samples					Mean Score Value (± SD)
	Score 1	**Score 2**	**Score 3**	**Score 4**	**Score 5**	
Group 1	1	6	3	0	0	2.20 ± 0.632
Group 2	2	5	1	2	0	2.30 ± 1.059
Group 3	1	3	1	5	0	3.00 ± 1.155
Group 4	0	0	0	0	10	5.00 ± 0.000

The score values of dentin tubule occlusion in the study groups (group 1: Karex toothpaste, group 2: Biorepair Plus Sensitive toothpaste, and group 3: Dr. Wolff's Biorepair toothpaste) and in the control group (group 4).

The mean values of carbon, calcium, phosphorus, and oxygen ion concentration (wt%) are presented in Table 3. Carbon ion concentration in group 3 was significantly higher when compared to group 2 and group 1 ($p < 0.05$, Table 4). Statistically significant results were also recorded when comparing carbon ion concentration in the study groups and the control group ($p < 0.05$, Table 4). Significantly increased calcium ion concentration was recorded in the study groups when compared to the control group ($p < 0.05$, Table 4). In group 3, significantly lower calcium concentration was observed when compared to group 2 ($p < 0.05$, Table 4). Phosphorous ion concentration was significantly higher in the study groups when compared to the control group ($p < 0.05$, Table 4). Significantly lower phosphorous ion concentration was recorded in group 3 when compared to group 1 and group 2 ($p < 0.05$, Table 4). Oxygen ion concentration in the study groups was significantly higher when compared to the control group ($p < 0.05$, Table 4). Also, statistically significant differences were recorded when comparing the values of tubule closure in the study groups and the control group ($p < 0.05$, Table 4). There was no significant difference when comparing the values of dentin tubule occlusion in the study groups (p values > 0.05, Table 4).

Table 3. Mean values and standard deviation of carbon, calcium, phosphorus, and oxygen ion concentration (wt%) in control and study groups.

	Mean Value of Ion Concentrations (wt%) ± Standard Deviation			
	Carbon	**Calcium**	**Phosphorus**	**Oxygen**
Group 1	12.89 ± 7.64	22.40 ± 6.80	11.87 ± 2.76	48.32 ± 4.29
Group 2	10.58 ± 7.32	24.14 ± 3.86	12.96 ± 2.06	48.62 ±1.88
Group 3	23.73 ± 10.57	18.18 ± 6.50	9.58 ± 2.99	46.26 ± 3.71
Group 4	38.60 ± 6.600	13.15 ± 3.38	6.68 ± 1.87	41.54 ± 3.36

Carbon, calcium, phosphorous, and oxygen ion concentrations in the study groups (group 1: Karex toothpaste, group 2: Biorepair Plus Sensitive toothpaste, and group 3: Dr. Wolff's Biorepair toothpaste) and in the control group (group 4).

Table 4. Statistical test results when comparing the concentration of carbon, calcium, phosphorus, and oxygen ion concentration and the dentinal tubule occlusion score in groups.

Ion Concentration	Levene Test		Kruskal-Wallis Test		Post-Hoc LSD Test		
	Statistics	*p* Value	Chi-Square	*p* Value	Statistically Significant Results		*p* Value
Carbon	0.855	0.473	24.464	0.000 *	Group = 1	Group = 3	0.005 *
					Group = 1	Group = 4	0.000 *
					Group = 2	Group = 3	0.001 *
					Group = 2	Group = 4	0.000 *
					Group = 3	Group = 4	0.000 *
Calcium	1.843	0.157	8.333	0.000 *	Group = 1	Group = 4	0.000 *
					Group = 2	Group = 3	0.018 *
					Group = 2	Group = 4	0.000 *
					Group = 3	Group = 4	0.043 *
Phosphorous	1.364	0.269	12.665	0.000 *	Group = 1	Group = 3	0.045 *
					Group = 1	Group = 4	0.000 *
					Group = 2	Group = 3	0.004 *
					Group = 2	Group = 4	0.000 *
					Group = 3	Group = 4	0.012 *
Oxygen	1.169	0.335	9.055	0.000 *	Group = 1	Group = 4	0.000 *
					Group = 2	Group = 4	0.000 *
					Group = 3	Group = 4	0.004 *

Dentinal Tubules Occlusion Score	Kolmogorov Smirnov Test	Mann-Whitney Test		
	p Value	Statistical Results		*p* Value
	0.000	Group 1	Group 2	0.967
		Group 1	Group 3	0.092
		Group 1	Group 4	0.000 *
		Group 2	Group 3	0.173
		Group 2	Group 4	0.000 *
		Group 3	Group 4	0.000 *

Statistical test resuls of ion concentrations and dentinal tubule occlusion score comparison between the study and control groups. * statistically significant differences between groups.

4. Discussion

The current in vitro study aimed to establish the degree of dentinal tubule occlusion and mineral deposition on the dentin surface when using three different toothpastes containing n-HAp as an active ingredient. In our study none of the three n-HAp toothpastes determined complete occlusion of the dentinal tubules. Previous studies also showed partial closure of the dentinal tubules after an acid attack when using Biorepair Plus Sensitive toothpaste [32]. A study that tested n-HAp toothpaste Renamel (Sangi, Tokyo, Japan) had also demonstrated partial occlusion of dentinal tubules. Only one study reported total closure of dental tubules when an experimental pure n-HAp desensitizing toothpaste was tested [13].

Regarding the occlusion of dentinal tubules when comparing the toothpaste containing nano-HAp crystals to other products, the results of previous studies are contradictory. The toothpaste containing 1% n-HAp crystals led to a significantly higher percentage of tubule occlusion when compared to proargin technology [9]. Toothpastes containing 2 wt% n-HAp determined a reduction of DH in patients as a result of enamel surface restoration and dentinal tubule closure [21,33,34]. Some studies confirmed the efficacy of n-HAp dentifrices in achieving tubule occlusion [35]. On the contrary, in other studies there was no statistical difference between n-HAp products (Biorepair Plus) and Pronamel (Sensodyne) regarding the protection against an acid attack [29]. It was demonstrated that polishing the dentin surface with n-HAp led to partial tubular closure, while 30% S-PRG (surface pre-reacted glass-ionomer) filler-containing paste determined complete tubular closure [36].

In our study, SEM investigation of dentin samples treated by all three n-HAp toothpastes had shown mineral deposition that occluded the dentinal tubules and covered the intertubular dentin. Similar precipitate layer formation on the dentin surface was reported in previous studies that tested 10% and 15% n-HAp solutions [37]. Efficient dentinal tubule occlusion and mineral crystal growth on the dentin surface and into the dentinal tubules due to application of an n-HAp desensitizer was highlighted in a recent study [38]. Our study also reported increased calcium, phosphorous, carbon, and oxygen ion concentrations when n-HAp toothpastes had been applied on the dentin surface. In the remineralization process it was shown that n-HAp may act as a template in mineral crystal nucleation and growth to form a structure similar to dentin. Calcium and phosphate ions from the environment may be attracted on the dentin surface during the precipitation process and will fill the vacant places in the crystal structure [39]. Some previous studies have shown that toothpastes containing $ZnCO_3$/n-HAp or n-HAp have similar enamel and dentine remineralization capacity, and the remineralization potential was higher when compared to fluoride toothpastes [20]. On the contrary, another study that compared the remineralization capacity of HAp toothpaste to fluoride and bioactive glass toothpastes concluded that the effectiveness of the remineralization process was lower for HAp products than for fluoride toothpaste [33].

To our knowledge, there is no study in the scientific literature to evaluate Karex toothpaste efficacy on dentinal tubule occlusion and very few articles analyzed the efficacy of Biorepair Plus and Dr. Wolff's Biorepair toothpastes only in comparison with other desensitizing dentifrices. In our study there was no statistically significant difference in tubular occlusion between the study groups, so our null hypothesis was not rejected. This is in accordance with the studies of Tschoppe et al. [19] and Pei et al. [13] which have demonstrated that different n-HAp toothpastes have similar capacities in the remineralization process [13,19]. However, Tschopee et al. did not use the toothpastes tested in the present study and performed the experiment on bovine dentin specimens.

It is difficult to compare the results recorded in different studies due to different methodology of sample preparation and application technique. The present study simulates an acid attack using 40% citric acid in order to obtain the sensitive tooth dentin pattern. In our product application protocol, the dentin samples were brushed using a tooth brushing machine and this might preclude the probability of applying different forces on the manual toothbrush handle. One limitation of our study was that the dentine sample was obtained by cutting the tooth crown in the middle third. DH usually involves exposure of the cervical dentinal tubules, which have a number, diameter, and orientation that are different from those of the middle part of the crown. Therefore, the deposition pattern of the crystals might be different in vivo compared to our experiment. Another limitation was the evaluation of the toothpaste's action in the absence of any acidic attack between tooth brushing, which normally occurs in the oral cavity. Furthermore, the samples were stored in artificial saliva between tooth brushing sessions, but neither buffer effect of saliva nor enzyme activity were simulated. Further clinical studies are needed to evaluate the action of commercial n-HAp toothpaste on DH treatment.

5. Conclusions

Toothpastes containing n-HAp determined a significant closure of dentinal tubules and a significant increase of mineral deposition on the dentin surface. All three tested toothpastes showed similar results regarding the degree of tubule closure. There was a varying degree of differences in calcium, phosphate, carbon, and oxygen ion concentrations among the three tested toothpastes.

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