ORIGINAL PAPER

IN VIVO EVALUATION OF ELECTROPHORESIS-AIDED RE-MINERALIZATION ON SURFACE TOPOGRAPHY AND CHEMICAL COMPOSITION OF DE-MINERALIZED ENAMEL: AN *IN VIVO* ANIMAL MODEL STUDY

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ABSTRACT

Introduction: The most mineralized and rigid tissue in the human body is tooth enamel. Enamel, the outermost covering of teeth, is made of hydroxyapatite, which is arranged in a well-organized prism structure to absorb loads. De-mineralization occurs when the most widely available and soluble material is removed from the enamel rods' periphery and it is exposed to acids, increasing the enamel's surface porosity, and allowing more acids to diffuse into the tissue and lose mineral ions.

Objectives: To evaluate whether an electrophoresis-aided system improved re-mineralization of de-mineralized enamel more than a traditional technique in rabbits with initial enamel lesions using different re-mineralizing agents.

Material and methods: Initial enamel lesion was produced through acid etching, and three re-mineralizing agents, such as nano-hydroxyapatite, casein phospho-peptide amorphous calcium phosphate, and bioactive glass, were applied to labial surface using traditional or electrophoresis-aided techniques. Surface topography and chemical composition were evaluated using environmental scanning electron microscope/energy dispersive X-ray spectrometer at baseline, after de-mineralization, and after re-mineralization, at different time periods, such as 2 weeks and 5 weeks for a traditional technique, and 3 hours and 5 hours for an electrophoresis-aided technique.

Results: Environmental scanning electron microscope showed that re-mineralizing agents enhanced re-mineralization of surface topography following different application periods. Energy dispersive X-ray spectrometer results revealed that for the traditional technique, there was no statistically significant difference between mean Ca : P ratio values of the three materials at 2 and 5 weeks. For the electrophoresis-aided technique, nano-hydroxyapatite showed the highest effect, with the most insignificant effect demonstrated in bioactive glass at both 3 and 5 hours.

Conclusions: Applying different re-mineralizing agents significantly influences enamel topography and chemical composition, and accelerate the effect of re-mineralizing agents. Moreover, the dynamics of re-mineralizing agents might be accelerated by electrophoresis.

Key words: electrophoresis, enamel re-mineralization, ESEM/EDX.

J Stoma 2024; 77, 2: 118-128
DOI: https://doi.org/10.5114/jos.2024.139909

JOURNAL OF STOMATOLOGY
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Received: 11.08.2023 • Accepted: 03.11.2023 • Published: 29.05.2024

INTRODUCTION

The most mineralized and rigid tissue in the human body is tooth enamel. Enamel, the outermost covering of teeth, is made of hydroxyapatite (HA), which is arranged in a well-organized prism structure to absorb loads [1]. De-mineralization occurs when the most widely available and soluble material is removed from the enamel rods' periphery and it is exposed to acids, increasing the enamel's surface porosity, and allowing more acids to diffuse into the tissue and lose mineral ions [2]. Partially de-mineralized HA crystals in teeth can enlarge to their original size if they are exposed to oral conditions, which encourage re-mineralization, since de-mineralization is reversible [3]. With the aid of calcium and phosphate ions delivered from an external source to the teeth, which induce ion precipitation within de-mineralized enamel and re-introduce net minerals, non-cavitated lesions can be repaired [4]. Many materials have been developed to halt the spread of caries or to further re-mineralize the lesion, alternatives to fluoride, such as nano-hydroxyapatite (n-HAP), casein phosphopeptide (CPP), and bioactive glass (BAG). One of the most biologically active and biocompatible materials is n-HAP. n-HAP has been widely used in dental research to biomimetically repair damaged enamel owing to its structural and chemical similarities with enamel minerals [5]. In the form of a topical nano-complex cream, casein phospho-peptide amorphous calcium phosphate (CPP-ACP) that is ACP complexed with milk protein, has a multifaceted anti-cariogenic impact and the ability to re-mineralize teeth [6]. In addition to maintaining high concentrations of calcium and phosphate ions, it stimulates re-mineralization of carious lesions by maintaining the minerals in a supersaturated form [7]. One of the proposed re-mineralizing agent is bio-active glass that can be used to re-mineralize teeth structures [8]. In an aqueous environment, bio-glass quickly begins to exhibit surface reactions, which result in cation exchange and formation of a crystalline layer from precipitation of calcium-phosphate [9]. It is noteworthy that the rate of precipitous growth is very slow, and may limit the effectiveness of these materials [10]. Electrophoresis, including ionophoresis, can transport ions more rapidly through a gel or solution than diffusion alone, and can be used to accelerate HA formation [11]. Furthermore, it was claimed that electrophoresis can impose a specific directionality to ion migration using a little amount of direct electrical current and a direct electric field [12].

The null hypothesis of the current research was that the electrophoresis-aided re-mineralization technique and the traditional technique will not differ significantly from one another.

OBJECTIVES

This *in vivo* animal study aimed to evaluate whether an electrophoresis-aided system improved re-mineralization of de-mineralized enamel more than a traditional technique in rabbits with initial enamel lesions using different re-mineralizing agents.

MATERIAL AND METHODS

The following materials were used in the study:

1. CPP-ACP re-mineralizing agent in the form of tooth mousse (GC Tooth Mousse, batch number: 210408C, lot No.: 04008181GC; International, Itabashi-Ku, Tokyo, Japan).
2. n-HAP re-mineralizing agent in the form of toothpaste (Apagard Royal toothpaste, batch number: 120-82-4101; Sangi, Co., Ltd., Japan).
3. Bioactive glass re-mineralizing agent in the form of toothpaste (BioMin C toothpaste, batch number: PMTB0039; BioMin Technologies Ltd., Bielefeld, Germany).
4. De-mineralizing agent in form of 37% phosphoric acid used for enamel de-mineralization (N-Etch, batch number: Z203PNM; Ivoclar, Vivadent, AG, Switzerland).

SAMPLE SIZE

According to a previous study by Zhang *et al.* [11], ~7 teeth in each sub-group were sufficient to detect an effect size of 0.51 at a power (1 – β error probability) of 0.8, and using a two-sided hypothesis test with significance level (α error probability) of 0.05.

TEETH GROUPING

According to re-mineralizing process, 84 teeth were randomly divided into two equal major groups (n = 42) (electrophoresis and traditional), and then classified into three equal sub-groups (n = 14) according to re-mineralizing agent used: n-HAP N, CPP-ACP C, and BAG B. Each sub-group was further divided into two equal divisions (n = 7), according to re-mineralization period: traditional re-mineralization sub-groups were divided into 2 weeks (W2) and 5 weeks (W5), while electrophoresis re-mineralization sub-groups were divided into 3 hours (H3) and 5 hours (H5).

INTERVENTION

New Zealand rabbits (4-month-old males, weighting approximately 2 kilograms [kg]) were anesthetized by intra-muscular injection in the quadriceps femoral muscle with 3.3 centimeters (cm) of Xyla-Ject solution at a concentration of 30 milligram (mg)/kg. Throughout the experiment, an additional dose of 10 mg/kg was provided (as required) to maintain the rabbits under anesthesia [13]. Ethical board of the local institution autho-

rized this study (approval code: 120192/3/32). All animal experimental procedures were performed according to the protocol of the Canadian Counion Animal Care and in coherence with the three Rs (replacement, reduction, reinforcement) of animal ethics [14]. Also, reporting of in vivo experiments (ARRIVE) guidelines were followed. There was no mortality during the study period.

SAMPLE PREPARATION

Maxillary incisors were acid-etched for one minute with 37% phosphoric acid (Ivoclar) to produce chalky white appearance with white spot lesions (WSLs) that mimic caries, and then rinsed with large amount of de-ionized water [15].

ANIMAL CODING FOR RE-MINERALIZATION TECHNIQUES

Identification of animals was done randomly to distinguish between them during the application of materials. Coding was performed by painting the inner ears of each rabbit using permanent marker (black color for n-HAP, red color for CPP-ACP, and green color for BAG).

SURFACE TREATMENT

All re-mineralizing agents' pastes included were commercial products. For the traditional technique applied to de-mineralized enamel with a micro-brush on the labial surface of de-mineralized specimens, brushing procedures were carried out in each group using a soft toothbrush (Oral B, Procter and Gamble Co., Cincinnati, Ohio, USA) and minimal pressure was applied, three time daily, every eight hours with undiluted toothpaste (approximately 1 gram [g]) for one minute. Specimens were cleaned with de-ionized water for 15 seconds following each brushing procedure. This procedure was repeated daily for 2 and 5 weeks [16, 17]. For the electrophoresis technique, a specialized mold was created with an entrance cavity on the labial surface. Electrophoresis apparatus used a two-electrode setup, with anode linked to the rabbit's skin and cathode inserted into a loaded custom-made mold with CPP-ACP, n-HAP, and BAG. Electric current was applied for 3 or 5 hours. Maxillary incisors were extracted after each re-mineralization procedure [11].

EVALUATION TECHNIQUE

The specimens were examined using environmental scanning electron (ESEM) device (FEI Company, Eindhoven, Netherlands) that was set to run in the back-scattered electron mode at 1000x magnification using ESEM, model Quanta 250 field emission gun (FEG), with accelerating voltage of 30 kilovolt (KV), magnification 14× up to 1,000,000.

Surface analysis was performed on evaluation specimens, including minerals' proportion, their presence and absence, particularly calcium (Ca), phosphorus (P), and fluoride, using SEM in combination with an energy-dispersive X-ray (SEM/ EDX) microscope to 10 KV, and equipped with a detector and Xplore (XP3) pulse processor (Oxford Instruments X-ray micro-analysis, Oxford Super ATW). The specimens were subjected to the electron beam with take-off angle of 35°. Spectroscopic pixel energy calibration (SPEC) vision-integrated acquisition system was used to collect EDX spectrum images.

RESULTS

ASSESSMENT OF SURFACE TOPOGRAPHY

ESEM IMAGES OF NORMAL AND DE-MINERALIZED ENAMELS

ESEM image of the rabbit's incisor tooth revealed a normal, smooth, and intact enamel surface (Figure 1A). Also, ESEM image of the rabbit incisor tooth showed a de-mineralized enamel surface after treatment with 37% phosphoric acid gel, and the surface of de-mineralized enamel presented uneven severe structural alterations. The enamel surface appeared uneven, and rough pores of different dimensions were observed on uneven surface of the enamel. Furthermore, surface cracks and fissures were visible (Figure 1B).

ESEM IMAGES OF TREATED ENAMEL AFTER TWO WEEKS USING TRADITIONAL TECHNIQUE

ESEM image showing the effect of re-mineralizing agents after two weeks revealed that the enamel crystals were not visible, but an area of calcified deposits (red arrow) was more evident and concentrated along porous defect (black arrow) over the old enamel (blue arrow). The n-HAP group had the highest re-mineralization and calcified deposits along the porous defect, followed by the CPP-ACP group, and the BAG group. Some porous areas remained in the BAG group (Figure 2).

ESEM IMAGES OF TREATED ENAMEL AFTER FIVE WEEKS USING TRADITIONAL TECHNIQUE

ESEM image showing the effect of re-mineralizing agents after five weeks revealed the original enamel and new crystals' layer, which increased in thickness and width, and their hexagonal became apparent. The enamel surface in the n-HAP group had the greatest re-mineralization and the most visible crystals, followed by the CPP-ACP

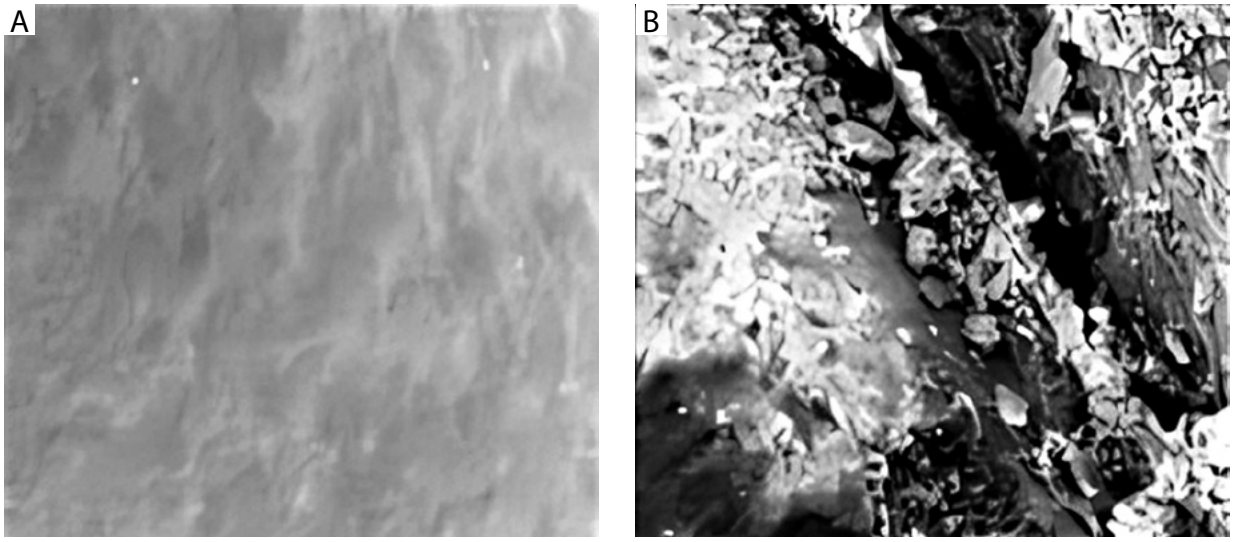

FIGURE 1. ESEM image at 500× for (**A**) normal enamel and (**B**) demineralized enamel

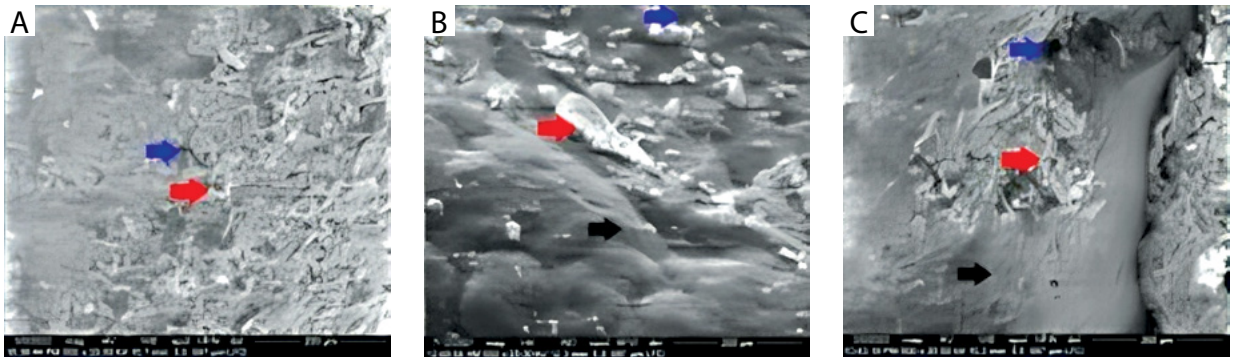

FIGURE 2. ESEM image at 500× for treated enamel with (**A**) n-HAP, (**B**) CPP-ACP and (**C**) BAG after 2 weeks

group. The BAG group samples had the least re-mineralization and the least visible crystals (Figure 3).

ESEM IMAGES OF TREATED ENAMEL AFTER THREE HOURS USING ELECTROPHORESIS TECHNIQUE

ESEM image showing the effect of re-mineralizing agents after three hours revealed different stages of the enamel surface formation and crystals fused (minerals globules). The enamel surface in the n-HAP group had maximum re-mineralization and the most apparent crystals than in other groups, followed by CPP-ACP, and BAG having the least amount of apparent crystals (Figure 4).

ESEM IMAGES OF TREATED ENAMEL AFTER FIVE HOURS USING ELECTROPHORESIS TECHNIQUE

ESEM image showing the effect of re-mineralizing agents after five hours revealed that the surface of de-mineralized enamel was fully re-mineralized and covered by a thick layer of new crystals. The enamel surface in the n-HAP group was fully re-mineralized, i.e., achieved maximum re-mineralization, and showed large crystals fused forming globules protuberances, followed by the CPP-ACP, while BAG had the least crystals apparent (Figure 5).

ASSESSMENT OF CHEMICAL COMPOSITION (EDX RESULTS) – CA : P MINERALS WT%

Numerical data were explored for normality by checking the distribution of data and using tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). All the data showed normal (parametric) distribution, and were presented as mean and standard deviation (SD) values. To study the effect of 2 different re-mineralization techniques using 3 different materials at 2 observation periods, ANOVA test was applied for comparison between the sub-groups. Three-way ANOVA test was used to investigate the effect of re-mineralization technique, time, material, and their interactions on mean hardness and Ca : P ratio. Bonferroni's post-hoc test was used for pair-wise comparisons when ANOVA test was significant. Pearson's

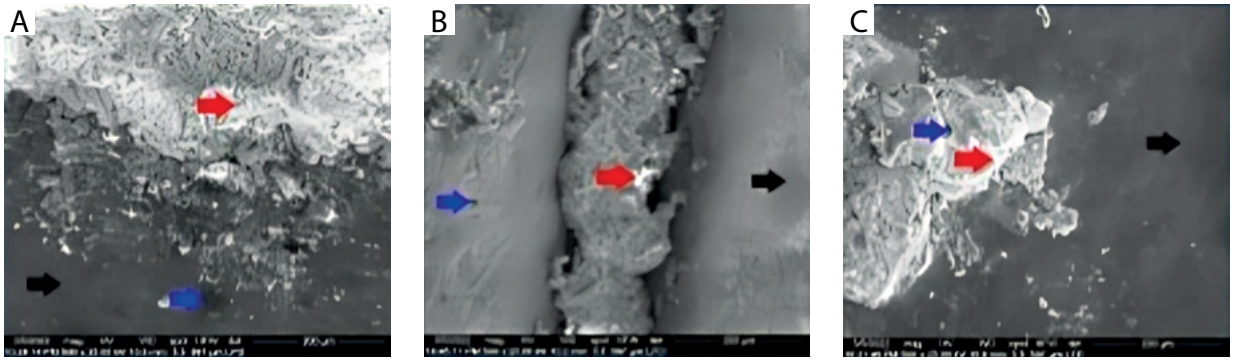

FIGURE 3. ESEM image at 500× for treated enamel with (**A**) n-HAP, (**B**) CPP-ACP and (**C**) BAG after 5 weeks

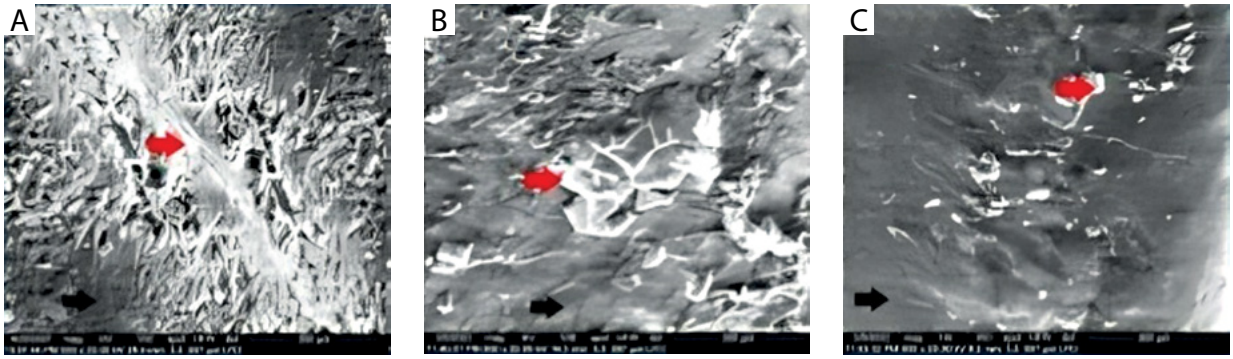

FIGURE 4. ESEM image at 500× for treated enamel with (**A**) n-HAP, (**B**) CPP-ACP and (**C**) BAG after three hours

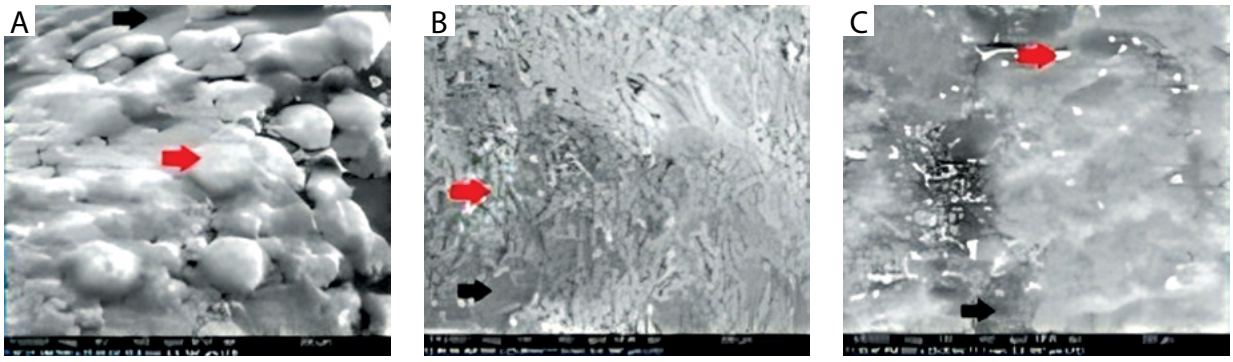

FIGURE 5. ESEM image at 500× for treated enamel with (**A**) n-HAP, (**B**) CPP-ACP and (**C**) BAG after five hours

correlation coefficient was applied to determine the correlation between Ca : P ratio and hardness. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with IBM SPSS Statistics for Windows, version 23.0 (IBM Corp.; Armonk, NY, USA).

COMPARISON BETWEEN MATERIALS

Using traditional technique whether at W2/H3 or W5/H5, there was no statistically significant difference between mean Ca : P ratio values of the three materials. Using electrophoresis at W2/H3, there was a statistically significant difference between mean Ca : P ratio values of different materials. Pair-wise comparisons between materials revealed that there was no statistically significant difference between n-HAP and CPP, and both showed statistically significantly higher mean Ca : P ratio compared with BAG. At W5/ H5 time points, there was a statistically significant difference between mean Ca : P ratio values of different materials. Pair-wise comparisons between materials revealed that there was no statistically significant difference between CPP and BAG, and both showed statistically significantly lower mean Ca : P ratio than n-HAP (Table 1, Figure 6).

TABLE 1. Mean, standard deviation (SD) values, and results of three-way ANOVA test for comparison between Ca : P ratio of three materials with different interactions of variables

Re-mineralization technique	Time point	n-HAP		CPP-ACP		BAG		*p*-value	Effect size (partial eta squared)
		Mean	SD	Mean	SD	Mean	SD		
Traditional technique	W2/H3	2.06	0.63	2.20	0.67	2.10	0.64	0.914	0.003
	W5/H5	2.50	0.77	1.75	0.53	1.80	0.55	0.052	0.079
Electrophoresis	W2/H3	2.20[a]	0.67	2.10[a]	0.64	1.36[b]	0.42	0.032*	0.091
	W5/H5	2.90[a]	0.89	1.80[b]	0.55	1.54[b]	0.47	< 0.001*	0.202

**Significant at p ≤ 0.05. Different superscripts indicate statistically significant differences between materials.*

TABLE 2. Mean, standard deviation (SD) values, and results of three-way ANOVA test for comparison between Ca : P ratio (VHN) at various times with different interactions of variables

Re-mineralization technique	Material	W2/H3		W5/H5		*p*-value	Effect size (partial eta squared)
		Mean	SD	Mean	SD		
Traditional technique	n-HAP	2.06	0.63	2.50	0.77	0.196	0.023
	CPP-ACP	2.20	0.67	1.75	0.53	0.189	0.024
	BAG	2.10	0.64	1.80	0.55	0.375	0.011
Electrophoresis	n-HAP	2.20	0.67	2.90	0.89	0.041*	0.056
	CPP-ACP	2.10	0.64	1.80	0.55	0.375	0.011
	BAG	1.36	0.42	1.54	0.47	0.608	0.004

**Significant at p ≤ 0.05.*

COMPARISON BETWEEN TIMES

Using traditional technique with n-HAP, CPP, and BAG, there was no statistically significant difference between mean Ca : P ratio values at different times. Using electrophoresis with n-HAP, the mean Ca : P ratio values at W2/H3 time points showed statistically significantly lower value than at W5/H5. While in CPP or BAG, there was no statistically significant difference between mean Ca : P ratio values at different times (Table 2, Figure 6).

COMPARISON BETWEEN RE-MINERALIZATION TECHNIQUES

Using n-HAP at W2/H3 or W5/H5, there was no statistically significant difference between mean Ca : P ratio values of the two re-mineralization techniques. Similarly, using CPP at W2/H3 or W5/H5, there was no statistically significant difference between mean Ca : P ratio values of the two re-mineralization techniques.

Using BAG at W2/H3, the traditional technique showed statistically significantly higher mean Ca : P ratio than electrophoresis. While at W5/H5, there was no statistically significant difference between mean Ca : P ratio values of the two re-mineralization techniques (Table 3, Figure 6). Figure 6 shows a chart representing mean and standard deviation values for Ca : P ratio with different interactions of variables.

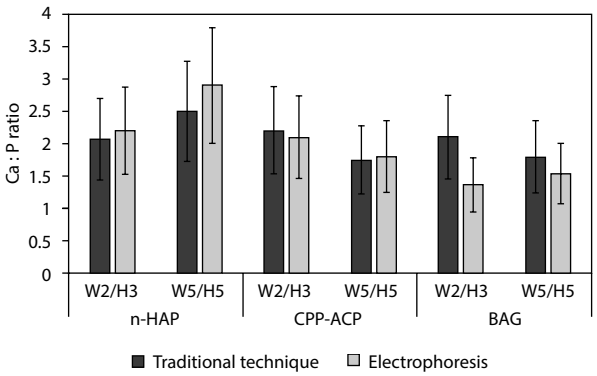

FIGURE 6. Bar chart represnting mean and standard deviation values for Ca : P ratio with different interactions of variables

DISCUSSION

The enamel surface layer often remains intact throughout sub-surface de-mineralization, creating white spot lesions as the earliest macroscopic sign of developing caries. At this stage without any intervention, it will eventually collapse into a full cavity. De-mineralized enamel crystals are electrically charged, which attract calcium and phosphate ions from re-mineralization solution. This helps to lower the surface energy of crystals, and promotes re-mineralization [11]. One of the goals of modem dentistry is to manage non-cavi-

TABLE 3. Mean, standard deviation (SD) values, and results of three-way ANOVA test for comparison between Ca : P ratio of two re-mineralization techniques with different interactions of variables

Material	Time	Traditional technique		Electrophoresis		*p*-value	Effect size (partial eta squared)
		Mean	SD	Mean	SD		
n-HAP	W2/H3	2.06	0.63	2.20	0.67	0.677	0.002
	W5/H5	2.50	0.77	2.90	0.89	0.239	0.019
CPP-ACP	W2/H3	2.20	0.67	2.10	0.64	0.775	0.001
	W5/H5	1.75	0.53	1.80	0.55	0.883	0.0001
BAG	W2/H3	2.10	0.64	1.36	0.42	0.034*	0.061
	W5/H5	1.80	0.55	1.54	0.47	0.452	0.008

**Significant at $p \leq 0.05$.*

tated white spot lesions and to prevent further disease progression [18]. Fluoride treatment has been demonstrated to re-mineralize white spot lesions, although it is effective in the first 10 to 30 μm of the lesion, necessitating the development of alternate treatments to enable re-mineralization of deeper layers [19]. Re-mineralization of early carious lesions may be accomplished using a range of currently available agents, including BAG, CPP ACP, and nano-hydroxyapatite.

Using human teeth has limitations, as it is hardly possible to provide the proper number of healthy teeth with suitable quality for research. Therefore, scientists are exploring a suitable substitute for dental research. Mouses, rabbits, and guinea pigs are laboratory animals from the rodent category [20]. For the current study, New Zealand rabbits were chosen as the animal model because of their short lifespan, larger tooth size, and pulp tissues, which are similar to those found in humans [21].

Phosphorus acid gel rather than solution applied on teeth is typically preferred because it is more viscous, and therefore stays in place longer, allowing for an extended contact time with the enamel. This increases the effectiveness of the acid in etching the enamel and creating microscopic pores, which are necessary for re-mineralizing agents in the toothpaste to penetrate and strengthen the teeth. Additionally, phosphorus acid gel is less likely to drip or run off the teeth, making it easier to apply and control. Sorozini suggested using 37% phosphoric acid that was deemed adequate because it mimicked oral circumstances and simulated early enamel lesion [22]. Artificial caries, such as the lesions made for this study, had lesions depths of 100-120 micrometer (μm), simulating a naturally occurring carious lesion in the oral cavity that has been active for three months [16]. In attempting to imitate daily brushing habits that would occur in real-life conditions, the specimens were given three daily brushing [16]. A digital scale was used to quantify the volume (1 gram), and water spray was then utilized to remove the gel and stimulate a condition in the oral cavity [23].

The re-mineralization effect appears after 3 hours to replace the water content of enamel with minerals [24], where the re-mineralization process is still in its early stages and calcium phosphate ions are just beginning to deposit on the enamel surface. While at 5 hours, ideally oriented and ordered mineralization pattern is accomplished [11]. Regarding two weeks' time, it is the accepted period according to many studies to test re-mineralization, because it is the amount of time for the teeth to show signs of re-mineralization. At five weeks, the re-mineralization process is typically completed. The mineralization in a 5 μm de-mineralized region starts 2 weeks after treatment, and complete re-mineralization can be achieved after 5 to 8 weeks [10].

In order to provide more comprehensive data, both qualitative and quantitative approaches were applied to correlate the histological appearance of the enamel surface during the de-mineralization/re-mineralization process, with quantitative data of the calcium and phosphate levels in the enamel. An environmental scanning electron microscope (ESEM) coupled with energy dispersive analysis X-ray (EDAX) were employed in the study for ultra-morphological analysis and non-destructive qualitative evaluation, and to record structural changes of the enamel surface for the treated surfaces of the enamel, according to Hegde and Moany study [25]. Results of this study revealed that the normal enamel surface of a rabbit showed a smooth surface while after enamel de-mineralization, ESEM images presented uneven, rough irregular surface, exposed crystals, open enamel prism, scattered leached-out minerals, and severe structural damage (Figure 1). After the application of re-mineralizing agents, such as n-HAP (N), CPP-ACP (C), and Bioactive glass (B), ESEM showed precipitation and deposition of Ca and phosphate (PO_4) nano-particles with different thicknesses on enamel surfaces and a significant increase in crystals, more than de-mineralized specimens.

FOR TRADITIONAL RE-MINERALIZATION TECHNIQUE

In the present study, the normal enamel surface of a rabbit showed a smooth surface while after enamel de-mineralization, ESEM images demonstrated uneven, rough irregular surface, exposed crystals, open enamel

prism, scattered leached-out minerals, and severe structural damage (Figure 1). After application of the remineralizing agents n-HAP, CPP-ACP, and Bio-active glass. ESEM showed precipitation and deposition of Ca and PO_4 nano-particles with different thicknesses on enamel surfaces with a significant increase in crystals more than demineralized specimens (Figures 2 and 3). ESEM showed finely fragmented particles covering the whole enamel surface, and they seemed to combine and form surface layer microstructure as well as globules and agglomerates. n-HAP revealed a highly significant difference from other groups, while no significant difference between BAG and CPP-ACP was observed, although five weeks showed more crystals deposition than two weeks in all groups (Figure 2).

The results of this study revealed that de-mineralized enamel's imperfections and micropores were filled by n-HAP crystals, which had settled onto the enamel surface. When evaluated from the perspective of the mechanism for nano-HA re-mineralization, the globules themselves appeared to be agglomerates of even smaller particles. During de-mineralization, acicular nano-HA crystals settled on the enamel surfaces and promptly filled-in defects and micropores, which is in agreement with Huang *et al.* [26] (Figure 2).

In an in situ investigation, an increased effect of a toothpaste containing n-HAP has been linked to the elevation of calcium concentrations, which resulted in re-mineralization of early caries lesions [27]. This is in agreement with Güçlü *et al.* [28] who reported that n-HAP shows more re-mineralization than BAG and CPP-ACP because it is soluble and its ions can be distributed to lesions, showing superior re-mineralization capacity. Previous results are consistent with Tschoppe *et al.* study [29]. On the other hand, these results are in disagreement with a study by Esteves *et al.* [30] who could not find significant difference in re-mineralizing using n-HAP; this may be due to differences in application time, technique, and testing periods.

In this study, there was an increase in minerals' deposition following CPP-ACP application due to the presence of calcium and phosphorus, which are vital components of enamel and are present in the form of a very insoluble compound (hydroxyapatite). CPP-ACP keeps these minerals soluble and biologically available on enamel surfaces [31]. This might be explained by the existence of a further extrinsic source of stable Ca and PO_4 ions, which might enhance saliva's inherent re-mineralization capacity by boosting diffusion gradients that encourage quicker and deeper substrate's re-mineralization [32].

In this study, the enamel re-mineralization potential of CPP-ACP and BAG was obvious in ESEM analysis at two weeks that showed that the BAG plug seemed more compact and closely adhered to the enamel surface than other plugs, which filled-in the crevices left by de-mineralization. Although BAG produced the larger and more angular deposit (Figure 2), it reduced the lesion depth. This finding was consistent with other studies by Preethee *et al.* [33] and Vashisht *et al.* [34].

For electrophoresis method, n-HAP revealed more re-mineralization than in other groups, while BAG demonstrated the lowest crystal deposition. Although at five hours period, more crystal deposition was seen than at three hours in all groups, according to SEM observations, the de-mineralized enamel was entirely re-mineralized at the completion of 5 hours (Figure 4).

n-HAP showed a large size of minerals arranged together forming enamel globules or protuberance and scattered large crystals more than in other groups, which demonstrated small size of minerals and small crystals with minor gaps; new and old phases of re-mineralization can appear. The n-HAP dentifrices consistently produced a greater amount of enamel crystals. The deposition of a precipitate layer in n-HAP was more than in CPP-ACP, while the lowest one was found in the BAG group due to its resistance to electricity.

Throughout H3 re-mineralization, the fragmentary crystals of de-mineralized enamel began to merge, resulting in original crystals extending, thickening, and closely packed on the surface of de-mineralized enamel. Also, in the region of original enamel, significant crystal growth was seen in addition to self-growing of de-mineralized enamel. With increased re-mineralization time, electrophoresis was also shown to be capable of successfully encouraging crystal formation. The surface of de-mineralized enamel was covered by a thick coat of crystals, as more and more new crystals were formed and accumulated. At the end of H5 re-mineralization, the de-mineralized enamel was fully re-mineralized, and its de-mineralized profile completely disappeared (Figure 5).

New crystals were created and accumulated to form a thick layer of crystals covering the surface of de-mineralized enamel in the prolonged re-mineralization period. The de-mineralized enamel was completely re-mineralized at the end of the 5-hour re-mineralization process, and its de-mineralized profile disappeared entirely in the n-HAP and CPP-ACP groups.

The significant impact might be attributable to the use of electric current flow, which has been demonstrated to improve material transport through enamel pores. As the fixed charge of enamel is mostly negative due to low (potential of hydrogen) pH, attack H^+ (hydrogen ion) outward of calcium that facilitate positive charge cations can penetrate enamel structure more rapidly [35].

During phoresies, the ions in toothpaste come in contact with cathode and move towards anode, where Ca in HAP crystals moves in the opposite direction; this movement facilitates the interaction of ions with calcium. Increased electric current helps the migration of calcium from n-HAP that also facilitates the release of PO_4, enabling the formation of apatite crystals; this effect appears after 3 hours. It was demonstrated that enamel's water content can be replaced with minerals in just three hours, as mentioned by Gan *et al.* [24]. This

is in agreement with Peng *et al.* [36] who used electric flow to help ions in HAP saturation, and accelerate early re-mineralization of early carious lesions of enamel.

CPP-ACP is a two-phase mixture that, when combined, interacts to produce ACP material, which precipitates onto tooth structure and raises calcium levels [37]. BAG at 3 hours showed an incomplete newly formed layer and incomplete crystals slightly resembled the native enamel. Although at 5 hours, ideally oriented and ordered mineralization pattern was partially accomplished; a result of the charge carrier's restriction in its freedom of movement within the network of glass. Instead, in response to applied alternating field, they displaced and polarized [38].

Regarding re-mineralization materials used in traditional technique, regardless of time periods, EDX results showed no statistically significant difference between mean Ca : P ratio values of the three materials. While using electrophoresis at W2/H3, there was a statistically significant difference between mean Ca : P ratio values of different materials. n-HAP and CPP both showed statistically significantly higher mean Ca : P ratio than BAG. At W5/H5, there was no statistically significant difference between CPP and BAG, and both showed statistically significantly lower mean Ca : P ratio than n-HAP, as demonstrated in Table 1 and Figure 6.

Improving the surface area of de-mineralized enamel may be attributable to the penetration of n-HAP nanoparticles, as their diameters, morphologies, and orientation of enamel apatite are similar. Since it fills up microscopic gaps and depressions on the enamel surface, n-HAP acts as a filler.

The other tested materials have re-mineralizing potential with different mechanisms of action. After the CPP-ACP application, there was a significant increase in the minerals ratio; this result is comparable with many other studies [25, 39] confirming the re-mineralizing capacity of CPP-ACP. Contrary to previous calcium phosphate technologies, the ions that BAG release from hydroxy carbonate apatite (HCA) directly without an intermediary amorphous calcium phosphate phase, could be its most probable mode of action [22].

Regarding re-mineralization periods using traditional technique whether with n-HAP, CPP, or BAG, there was no statistically significant difference between mean Ca : P ratio values at different re-mineralization periods. While using electrophoresis with n-HAP, the mean Ca : P ratio values at W2/H3 showed statistically significantly lower value than at W5/H5. While with CPP or BAG, there was no statistically significant difference between mean Ca : P ratio values at different periods, as presented in Table 2 and Figure 6.

As n-HAP is a highly conductive material, BAG is a low conductive material that might result from iontophoresis speeding up the flow of re-mineralizing ions into the most destructive sub-surface caries lesion [40].

The velocity of ion dissolution from the re-mineralization agent and the length of ionic contact with the substrate are crucial factors in the increased effectiveness of re-mineralization agents. When the exposure time lengthens, the ions from n-HAP dissolve, facilitating improved HA production [41].

The rapid diffusion of neutral ion pair (Ca, H, PO_4^{3-}), calcium and phosphate super-saturation result in increased mineral recovery in lesion's core. Additional PO_4^{3-} groups at the surfaces lead all surfaces to be negatively charged, and the variation is most likely due to varied configurations of the additionally charged ions, which make up the HAP lattice on each crystal plane. Surface charge is predicted to have a significant impact on inorganic and organic deposition processes as well as on structural evolution [41]. Ionic flux from the glass matrix and crystallization have been significantly impacted by changes in the BAG component with cation addition [42].

Furthermore, our results showed that there was no significant difference between both traditional and electrophoresis techniques, except for BAG at W2/H3. Traditional technique showed statistically significantly higher mean Ca : P ratio than electrophoresis, as presented in Table 3 and Figure 6. This may be due to ionic flux from glass matrix, as mentioned before [42].

Based on our results, the null hypothesis was partially accepted, since the electrophoresis-aided re-mineralization technique and the traditional technique did not differ significantly from one another, except for BAG.

CONCLUSIONS

Under the circumstance of the study and limitation of the materials used, we may conclude that the effects of re-mineralizing agents can be accelerated by electrophoresis, which can help strengthen de-mineralized enamel. The topography of the enamel surface is significantly influenced by the re-mineralizing agents. The application of the used different re-mineralizing agents enhances enamel re-mineralization (calcium and phosphorous contents), and various re-mineralizing agents' compositions have different effects on the enamel surface.

RECOMMENDATIONS

More investigation is required to determine how other materials interact with the electrophoresis technique, and to ascertain the effects of varied re-mineralization times, electric volts, or repeated application of materials using the electrophoresis technique. BAG was not the material of choice to be used with the electrophoresis technique.

APPLICABILITY OF METHODOLOGY FOR HUMANS

New Zealand rabbits have tooth anatomy and enamel composition similar to humans. This suggests that elec-

trophoresis would likely be just as effective in re-mineralizing cavities in humans as in rabbits. Electrophoresis is a non-invasive and relatively painless procedure. This makes it a particularly attractive option for treating early lesions in children and people with anxiety about dental procedures. Additionally, the electric current used in the electrophoresis-aided system is very low. It is unlikely to cause any harm to the patient. Using electrokinetic flows help the relevant ions for hydroxyapatite saturation (Ca, F, Na, and K) to be infiltrated into the enamel layer.

DISCLOSURES

1. Institutional review board statement: This study was conducted in accordance with all the provisions of the animal subjects oversight committee guidelines and policies of Faculty of Dental Medicine, Boys Branch, Cairo, Al-Azhar University, Egypt. The approval code for this study is: 120192/3/32.
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: The authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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