Evaluation of Electrophoresis-Aided Remineralization On the Surface Morphology Of Demineralized Enamel

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Abstract:

Aim: This in-vivo animal study aimed to evaluate whether an electrophoresis-aided system improved remineralization of demineralized enamel more than the traditional technique in rabbits with initial enamel lesions using different remineralizing agents. **Materials and Methods:** The initial enamel lesion was produced through acid etching, three remineralizing agents (nano-hydroxyapatite(n-HAP), casein phospho-peptide amorphous calcium phosphate (CPP-ACP), and bioactive glass (BAG)) were applied to the labial surface using traditional or electrophoresis-aided techniques. Surface topography was evaluated using ESEM at different periods. ESEM images were imported into Image J. software for further analysis. **Results:** ESEM showed that remineralizing agents enhanced remineralization of surface topography following different application periods. **Conclusion:** Applying different remineralizing agents significantly influences enamel topography. Further time has a significant positive impact on remineralization. Moreover, the dynamics of remineralizing agents might be accelerated by electrophoresis.

Keywords: In Vivo animal model, enamel remineralization, electrophoresis, image J. software.

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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 [1,2]. Demineralization occurs when the most widely available and soluble material is removed from the enamel rods' periphery when it is exposed to acids, increasing the enamel's surface porosity and allowing more acids to diffuse into the tissue and lose mineral ions from it [3]. One of the most biologically active and biocompatible materials is nano-hydroxyapatite (n-HAP). Nano-hydroxyapatite has been widely used in dental research to biomimetically mend damaged enamel because of its structural and chemical similarities with enamel minerals [4]. In the form of a topical nano-complex cream, casein phospho-peptide amorphous calcium phosphate (CPP-ACP), which is ACP complexed with milk protein, has a multifaceted anti-cariogenic impact and the ability to remineralize teeth [5]. One of the proposed remineralizing agent is bioactive glass, in an aqueous environment, bioglass quickly begins to exhibit surface reactions that result in cation exchange and the formation of a crystalline layer from the precipitation of calcium-phosphate [6]. Electrophoresis, including ionophoresis, can transport ions more rapidly and can be used to accelerate HA formation [7].

The null hypothesis of this research is that the electrophoresis-aided remineralization technique and the traditional technique will not differ significantly from one another.

Materials and methods:

1. **Casein phosphopeptide-Amorphous calcium phosphate (CPP-ACP)** remineralizing agent in the form of (tooth mousse paste, GC International, Itabashi- Ku, Tokyo, Japan).
2. **Nano Hydroxyapatite (n-HAP)** remineralizing agent in the form of (Apagard Royal toothpaste, Sangi, CO., Ltd., Japan).
3. **Bioactive glass** remineralizing agent in the form of (Biomin C toothpaste Bielefeld, Germany).

4. **Demineralizing agent** in form of 37% phosphoric acid was used for enamel demineralization (Ivoclar, Vivadent, AG).

Sample size: To study the effect of 2 different remineralization techniques using 3 different materials at 2 observation periods, an ANOVA test was used for comparison between subgroups. According to a previous study by Zhang et al (2018). (~7 teeth in each subgroup) 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 and a significance level (α error probability) of 0.05 for data.

Teeth grouping:

According to the remineralizing process, 84 teeth were randomly split into two equal major groups (n=42) electrophoresis or traditional, then classified into three equal subgroups (n=14) according to the remineralizing agent utilized, ***Subgroup N:*** was received n-HAP. ***Subgroup C:*** received CPP-ACP. ***Subgroup B:*** received BAG, and each subgroup was further divided into two equal divisions (n=7) according to the remineralization period for traditional remineralization subgroups which were divided into 2 weeks and 5 weeks while the electrophoresis remineralization subgroups were divided into 3 and 5 hours.

Intervention:

New Zealand rabbits (Males, 4-month-old, and weighting approximately 2 kg) were anesthetized by intramuscular injection in the quadriceps femoral muscle using 3.3 cm of Xyla-ject solution at a concentration of 30.0 mg/kg. Throughout the experiment, an additional dose of 10 mg/kg was provided as required to maintain the rabbits under anesthesia [8]. The ethical board of the Faculty of Dental Medicine, Boys, Cairo, Al-Azhar University authorized this study (120192\3\32). All animal experimental procedures and housing were considered and performed by a well-trained veterinarian according to the protocol of the Canadian

Counion Animal Care and in coherence with the three Rs (replacement, reduction, reinforcement) of animal ethics [9].

Sample Preparation:

The maxillary incisors were acid-etched for one minute with 37% phosphoric acid (Ivoclar) to produce chalky white appearance represent white spot lesions (WSLs) that mimic caries, then rinsed with large amount of deionized water [10].

Animal coding for remineralization techniques:

Identification of the 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 remineralizing agents' pastes were included as commercial products. For the traditional technique, applied to demineralized enamel with a micro brush on the labial surface of demineralized specimens, brushing procedures were carried out in each group using a soft toothbrush (Oral-B) [4] and minimal pressure three time daily every eight hours with undiluted toothpaste (approximately 1g) for one minute. The specimens were cleaned with deionized water for 15 seconds following each brushing procedure. The treatment procedure was repeated daily for 2 and 5 weeks [11,12]. For the application of the electrophoresis technique, a specialized mold was created with an entrance cavity on the labial surface. The electrophoresis apparatus used a two-electrode setup, with the anode linked to the rabbit's skin and the cathode inserted into a loaded custom-made mold with CPP-ACP, n-HAP, and BAG.

[4] (Procter and Gamble Co, Cincinnati, Ohio, USA)

The electric current was applied for 3 or 5 hours. The maxillary incisors were extracted after each remineralization procedure [13].

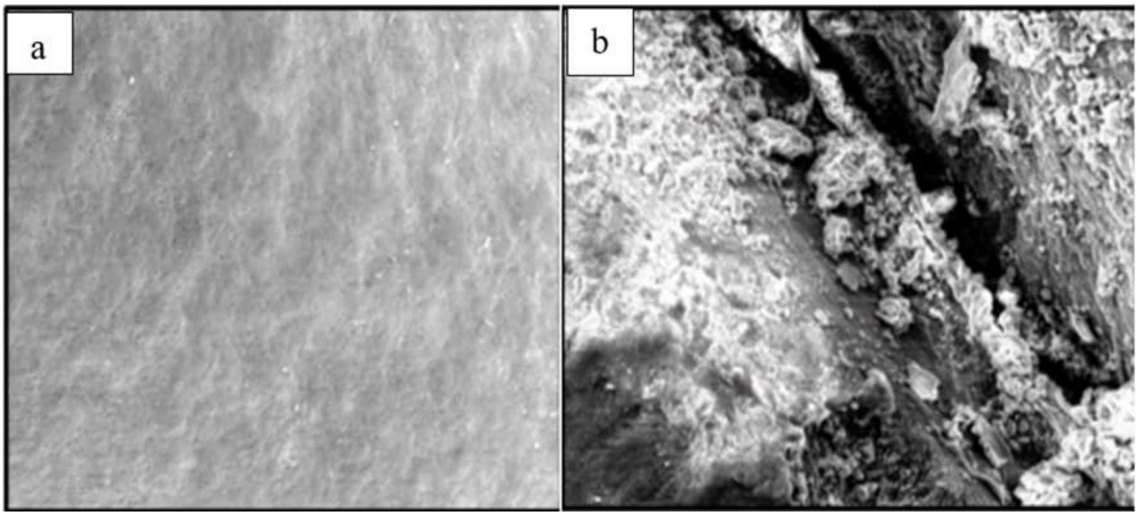
Evaluation technique:

The specimens were examined using the Environmental Scanning Electron (ESEM)[5] device, which was set to run in the backscattered electron mode at 1000X magnification. using ESEM Model Quanta 250 FEG (Field Emission Gun) with accelerating voltage 30 K.V., magnification 14x up to 1000000. ESEM images were imported into Image J. software for further analysis.

Results

- ESEM images of normal and demineralized enamel

ESEM image of the incisor tooth of the rabbit for normal enamel revealed a normal, smooth, and intact enamel surface, while the surface of demineralized enamel shows uneven severe structural alterations, rough, pores of different dimensions were observed also surface cracks and fissures were visible (Fig.).



[5] FEI- Company, Netherlands

Fig. 1: ESEM image at 500X for (a) normal enamel and (b) demineralized enamel.

- 3-D ESEM image of normal and demineralized enamel

3-D ESEM image of normal enamel revealed a smooth and an even surface in all different dimensions indicating the smooth surface area of enamel (where the x, y&z axis show normally as the Y axis represents the surface area of enamel, X represents the thickness of area and Z represent depth while color represent saturation of demineralization/remineralization area "increases redness increase saturation")

While for demineralized enamel using 37% phosphoric acid gel, revealed uneven, rough, pores of different dimensions (where the x-axis appears irregular and uneven, the y-axis indicates the thickness of pores areas, and z axis denotes the depth of demineralization area) (Fig.).

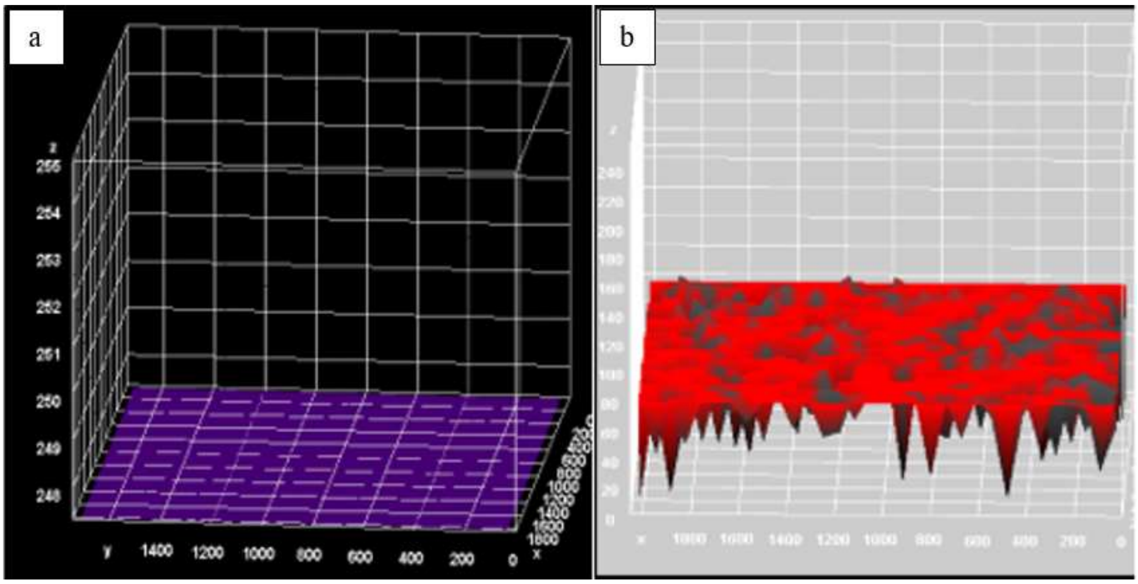


Fig. 2: Representative 3-D ESEM image of (a) normal enamel and (b) demineralized enamel.

- ESEM images of treated enamel with traditional technique after two weeks using different remineralizing agents:

ESEM image showing the effect of remineralizing agents after two weeks revealed that the enamel crystals were not discernible, but an area of calcified deposits (red arrow) were more evident and concentrated along porous defect (black arrow) over the old enamel (blue arrow). The enamel surface in the n-HAP group had maximum remineralization and calcified deposits along porous defect followed by CPP-ACP then BAG where still some porous appear (Fig.).

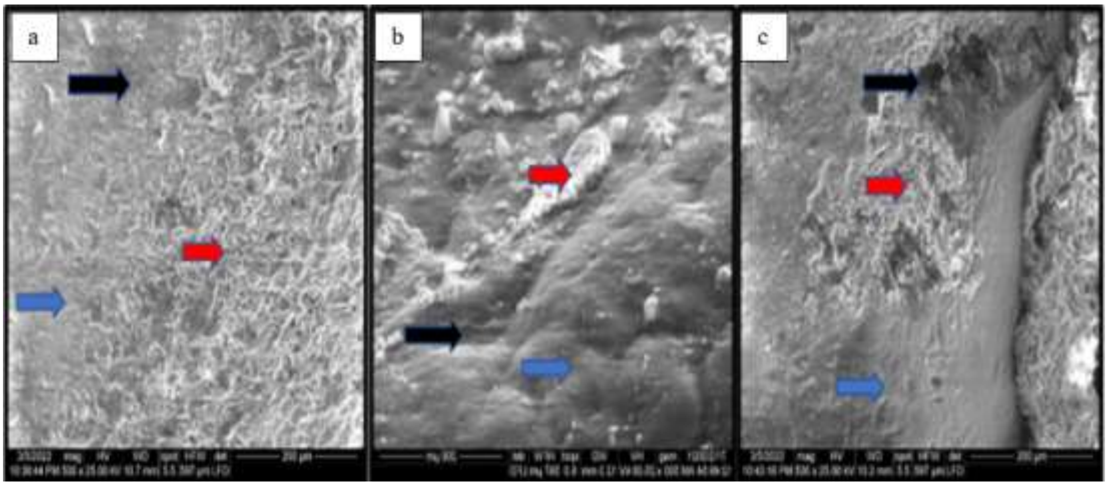


Fig. 3: ESEM image at 500X for treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after 2 weeks.

- 3-D ESEM image of treated enamel with traditional technique after 2 weeks using different remineralizing agents:

3-D ESEM image showing the effect of remineralizing agents after two weeks revealed that enamel surface becomes remineralized and old enamel covered with remineralizing crystals (represented by column bars). n-HAP revealed more mineralized surface as more column bars appear, more thickness, length of bars which denoting depth of mineralization and saturation of color which denoting saturation of remineralization followed by BAG, then CPP-ACP (Fig.).

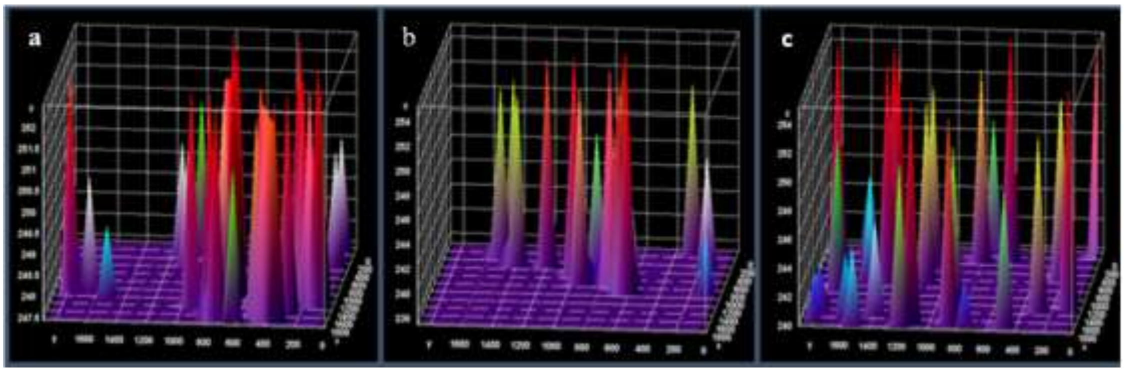


Fig. 4: Representative 3-D ESEM image of treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after 2 weeks.

– ESEM images of treated enamel with traditional technique after five weeks using different remineralizing agents:

ESEM image showed the effect of remineralizing agents after five weeks revealed the original enamel and new crystals layer which increase in thickness and width and their hexagonal shape become apparent. The enamel surface in the n-HAP group had maximum remineralization and more apparent crystals followed by CPP-ACP with the least one in the BAG group (Fig.).

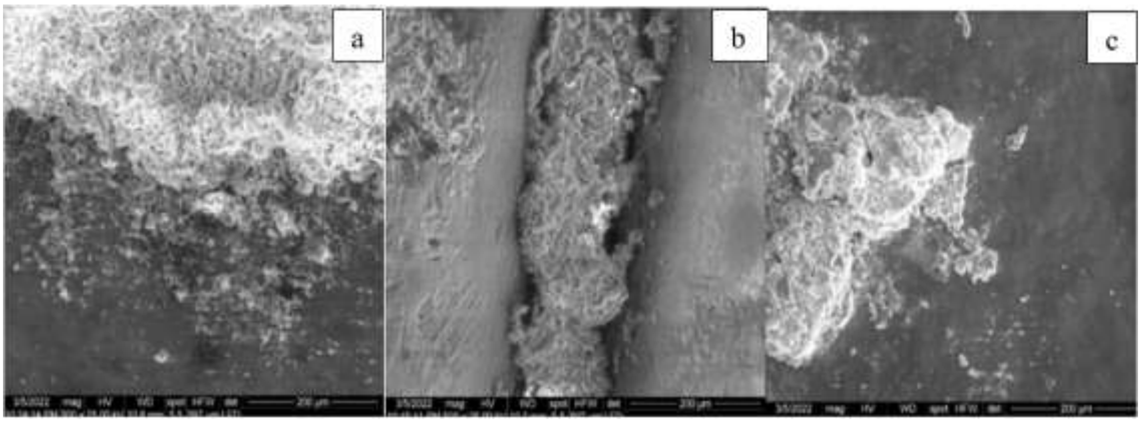
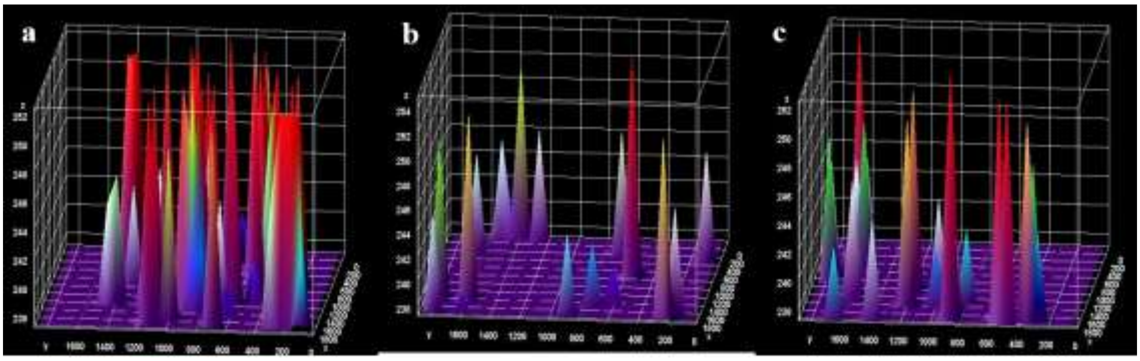


Fig. 5: ESEM image at 500X for treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after 5 weeks.

- 3-D ESEM image of treated enamel with traditional technique after five weeks using different remineralizing agents:

3-D ESEM image showing the effect of remineralizing agents after five weeks revealed increased enamel crystals in number and thickness. As more column bars appear which represent number of crystals, more thickness, length of bars which denoting depth of mineralization and saturation of color which denoting saturation of remineralization so n-HAP



revealed more mineralization enamel followed by BAG and then CPP-ACP group (

Fig.)

Fig. 6: Rrpresentative 3-D ESEM image of treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after 5 weeks.

– ESEM images of treated enamel with electrophoresis technique after three hours using different remineralizing agents:

ESEM image showed the effect of remineralizing agents after three hours revealed different stages of enamel surface formation and crystals fusion (minerals globules). The enamel surface in the n-HAP group had maximum remineralization and more apparent crystals than other groups, followed by CPP-ACP while BAG had the least apparent crystals (Fig.).

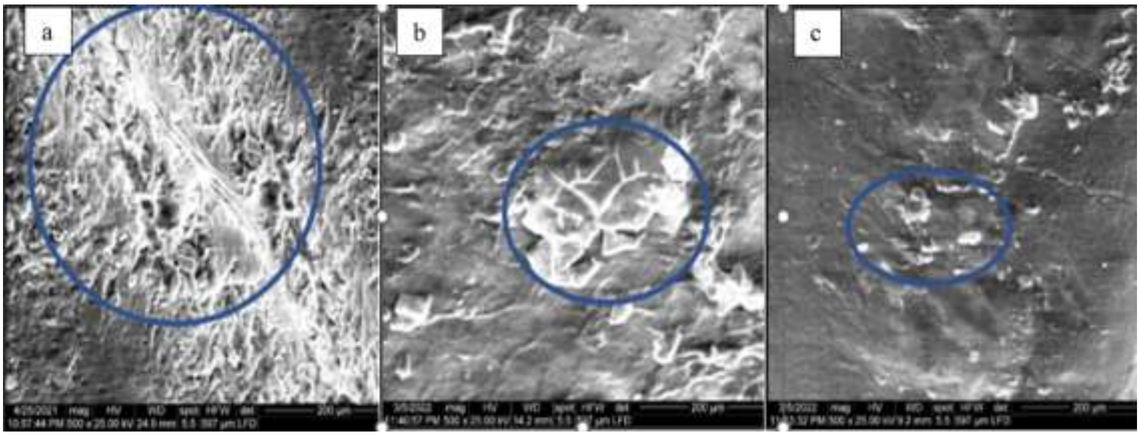


Fig. 7: ESEM image at 500X for treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after three hours.

3-D ESEM image of treated enamel with electrophoresis after three hours using different remineralizing agents:

3-D ESEM image showing th effect of remineralizing agents after three hours revealed that enamel crystals increased in number and thickness. as more column bars appear which represent the number of crystals, more thickness, length of bars which denoting depth of mineralization and saturation of color which denoting saturation of remineralization so n-HAP revealed more mineralization followed by CPP-ACP while BAG showed the least remineralization (Fig.).

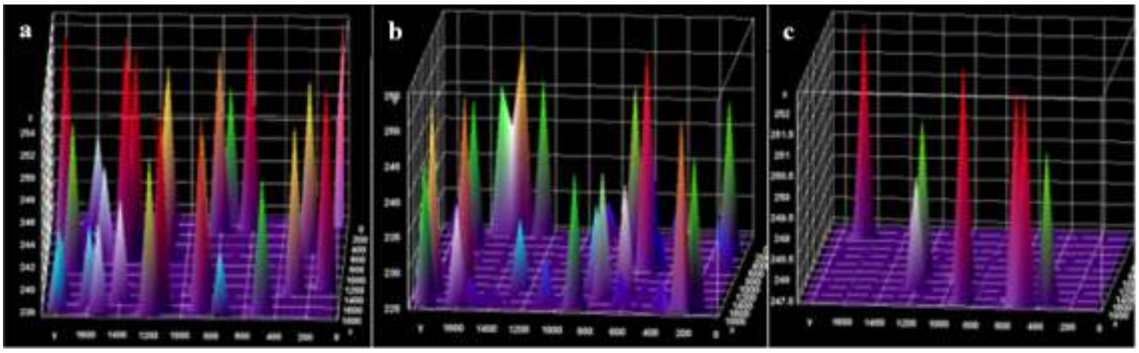


Fig. 8: Representative 3-D ESEM image of treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after three hours.

- ESEM images of treated enamel with electrophoresis technique after five hours using different remineralizing agents:

ESEM image showing the effect of remineralizing agents after five hours revealed that the surface of demineralized enamel was fully remineralized and covered by a thick layer of new crystals. The enamel surface in the n-HAP group was fully remineralized, had maximum remineralization, and showed large, fused crystals forming globules protuberances followed by CPP-ACP while BAG had the least apparent crystals (

Fig.).

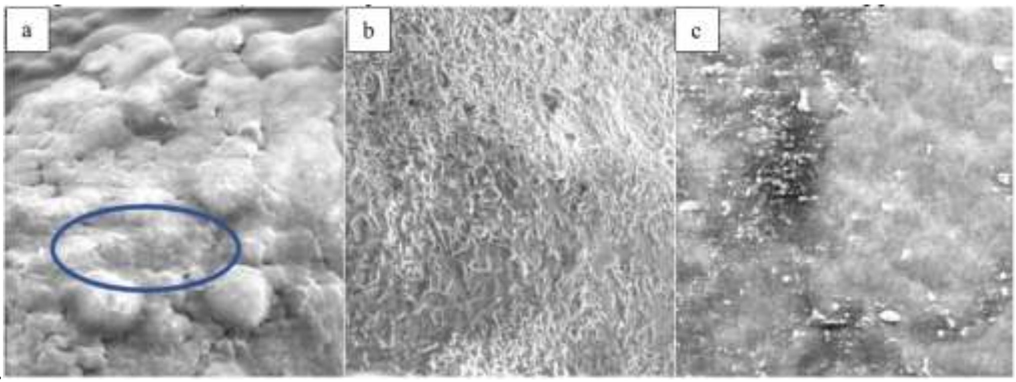


Fig. 9: ESEM image at 500X for treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after five hours.

- 3-D ESEM image of treated enamel with electrophoresis after five hours using different remineralizing agents:

3-D ESEM image showing the effect of remineralizing agents after five hours revealed that surface of demineralized enamel was fully remineralized and covered by a thick layer of new crystals revealing enamel crystals, as more column bars appear which represent number of crystals, more thickness, and length of bars which denoting depth of mineralization and saturation of color which denoting saturation of remineralization so n-HAP revealed maximum remineralization followed by CPP-ACP while BAG group showed the least remineralization (

Fig.).

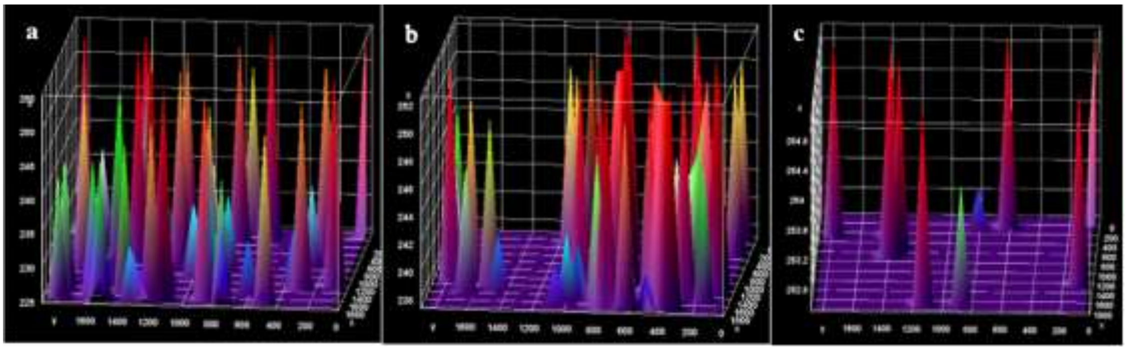


Fig. 10: Representative 3-D ESEM image of treated enamel with (a) n-HAP (b) CPP-ACP and (C) BAG after five hours.

Discussion

The equilibrium between the ongoing processes of demineralization and remineralization is so delicate, imbalances that persist for a long time typically result in early carious lesions [14]. The enamel surface layer often remains intact throughout subsurface demineralization, 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. Crystals in the demineralized enamel are electrically charged, allowing them to readily draw calcium and phosphate ions from the remineralization solution to lower surface energy [13]. Remineralization of early, non-cavitated lesions is one such treatment, as opposed to traditional treatment [15]. Fluoride treatment has been demonstrated to remineralize white spot lesions, although it is only effective in the first 10 to 30 mm of the lesion, necessitating the development of alternate treatments to enable the remineralization of deeper areas [16]. Remineralization of early carious lesions may be accomplished using a range of currently available agents including BAG, CPP ACP, and nano-hydroxyapatite.

The use of human teeth has limitations since it is difficult to obtain enough quantity of healthy teeth that are of sufficient quality for research, rabbits were chosen as the study's animal model because their pulp tissues were like those found in humans [17].

Sorozini suggested using 37% phosphoric acid, which was deemed adequate because it mimicked oral circumstances and simulating an early enamel lesion [18].

In attempting to imitate daily brushing habits that would occur in real-life conditions, the specimens were given three daily [11].

An environmental scanning electron microscope (ESEM) was employed in the study for ultra-morphological analysis and non-destructive qualitative evaluation and to record the structural changes of the enamel surface for the treated surfaces of enamel. in the same way as Hegde and Moany did in 2012 [19].

Results of this study revealed that, the normal enamel surface of the rabbit showed a smooth surface while after enamel demineralization the ESEM images showed uneven, rough irregular surface, exposed crystals, open enamel prism, scattered leached-out minerals, and severe structural damage. figure (1-2).

After the application of the remineralizing agents n-HAP (N), CPP-ACP (C), and Bioactive glass (B). ESEM showed precipitation and deposition of Ca & PO_4 nanoparticles with different thicknesses on enamel surfaces with a significant increase in crystals more than the demineralized specimens.

For traditional remineralization technique

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 although five weeks show more crystals deposition than two weeks in all groups.

Results of this study revealed that demineralized enamel's imperfections and micropores were filled by n-HAP crystals that had settled onto the enamel surface, when evaluated from the standpoint of a mechanism for nano-HA remineralization, the globules themselves appeared to be agglomerates of even smaller particles. During demineralization, acicular nano-HA crystals settled on the enamel surfaces and promptly filled in defects and micropores. This agrees with Huang et al 2009 [20] figure (3-4).

In an in-situ investigation, the increased effect of toothpaste containing n-HAP has been linked to the elevation of calcium concentrations, which results in the remineralization of early caries lesions [21]. This agrees with Guclu et al who reported that n-HAP shows more remineralization than BAG and CPP-ACP as it is soluble, and its ions can diffuse to lesions and show superior remineralization capacity [22]. The previous results are consistent with that research **by *Tschoppe et al., in 2011,* and Manchery et al., in 2019** who found that n-HAP is more effective than BAG [23-11]. On the other hand, those results were in disagreement with **Esteves et al., in 2017** [24] who could not find a remineralizing significant difference for n-HAP this may be due to differences in application time, technique, and testing periods.

In this study, there was an increase in the minerals deposition following CPP-ACP applied 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 [25]. This might be explained by the existence of a further extrinsic source of stable Ca and PO4 ions, which might enhance saliva's inherent remineralization capacity by boosting diffusion gradients that encourage quicker and deeper substrate remineralization [26].

In this study the enamel remineralization potential of CPP-ACP and BAG was obvious by using ESEM analysis at two weeks which showed the BAG plug seemed more compact and closely adhered to the enamel surface than other plugs that filled in the crevices left by demineralization. Although BAG produced the larger, more angular deposit, CPP-ACP produced smaller, more amorphous deposits figure (3-4). BAG reduces lesion depth; this finding was consistent with another research by Preethee et al., in 2011 and Vahid et al., in 2012 [27-28].

For electrophoresis method

n-HAP revealed more remineralized than other groups, while BAG showed the lowest crystal deposition. Although five hours show more crystal deposition than three hours in all groups (according to the SEM observations, the demineralized enamel was totally and entirely remineralized at the completion of 5H.) figure (7-10).

n-HAP shows a large size of minerals arranged together forming enamel globules or protuberance and scattered large crystals more than other groups which show small size of minerals, small crystals with small gaps, also new and old phases of remineralization may 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 the CPP-ACP while the lowest one was found in the BAG group due to its resistance to electricity.

Throughout 3H remineralization, the fragmentary crystals of demineralized enamel began to merge, resulting in the original crystals extending and thickening. With increased remineralization time, electrophoresis was also shown to be capable of successfully encouraging crystal formation, the surface of demineralized enamel is covered by a thick coating of crystals that have formed as more and more new crystals have formed and accumulated. At the end of 5 h remineralization, the demineralized enamel was fully remineralized, and its demineralized profile completely disappeared.

In agreement with the study of **Watanabe et al., in 2006** Complete mineral formation was achieved in 30 minutes using electrophoresis to precipitate hydroxyapatites [29].

The new crystal layer and the old crystal enamel were well delineated in the SEM micrographs of remineralized enamel following 3H remineralization. At the conclusion of the 3H remineralization, newly produced crystals were grouped and closely packed on the surface of the demineralized enamel. In the region of the original enamel, significant crystal growth was also seen in addition to the self-growing of demineralized enamel. The number of new crystals being created and accumulated to form a thick layer of crystals covering the surface of demineralized enamel has risen with the prolonged remineralization period. The demineralized enamel was completely remineralized at the end of the 5-hour remineralization process, and its demineralized profile vanished 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 pH, attack H+ and outward of ca that facilitate positive charge cations can penetrate enamel structure more rapidly [30].

During phoresies, the ions in toothpaste meet the cathode and move toward the anode where Ca in HAP crystals moves in the opposite direction, this movement facilitates the interaction of ions with calcium. Increased electric current facilitate the migration of Ca from n-HAP also facilitates the release of Po_4 which facilitates the formation of apatite crystals, also, the effect appears after 3H; it was demonstrated that enamel's water content can be replaced with minerals in just three hours as a mentioned by **Gan et al., in 2015** [31]. This agrees with **peng et al in 2019** [32] who uses electric flow to help ions for HAP saturation and accelerate early remineralization of early carious lesions of enamel.

The CPP-ACP is a two-phase mixture that, when combined, interacts to produce the ACP material, which precipitates onto tooth structure and raises calcium levels [33].

In BAG, at 3h showed an incomplete newly formed layer and incomplete crystals slightly resemble the native enamel Although at 5h ideal oriented and ordered mineralization pattern was accomplished partially a result of the charge carrier's restriction in its freedom of movement within the network of glass. Instead, in response to an applied alternating field, they displaced and polarized [34].

Conclusions

Under the circumstance of the study and limitation of the used materials:

1- The effects of remineralizing agents can be hastened by electrophoresis, which can help strengthen demineralized enamel.

2- The topography of the enamel surface is significantly influenced by the remineralizing agents.

3- Various remineralizing agent compositions have different effects on the enamel surface.

4- Time has positive effect on the remineralization process.

Recommendation

1. More investigation is required to determine how other materials interact with the electrophoresis technique.
2. To ascertain the effects of varied remineralization times, more research is necessary., electric volts, or repeated application of materials using the electrophoresis technique.

3- BAG was not the material of choice to be used with the electrophoresis technique.

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Conflict of Interest

The authors have no conflicts of interest to declare.

Data Availability

Datasets related to this article will be available upon request to the corresponding author.

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