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Comparative evaluation of remineralizing potential of four enamel remineralising agents using SEM-EDX – An in-vitro study

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A R T I C L E I N F O

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A B S T R A C T

Objective: Compare the remineralisation potential of four enamel remineralising agents on artificially demineralized enamel surface using Scanning electron microscopy- Energy dispersive x-ray.

Materials and methods: 75 extracted maxillary and mandibular premolars coated with acid-resistant nail varnish, were stored in demineralising solution for 96 h to produce artificial caries lesions. The samples were divided into 5 groups ($n = 15$), Control (Demineralized- No treatment), Group I samples were treated with casein phosphoprotein-amorphous calcium phosphate (CPP-ACP), Group II with self-assembling peptide (SAP-14), Group III with tri-calcium phosphate (f-TCP) and Group IV with Bioactive glass (BAG), respectively. The pH cycling model was followed for 21 days. The samples were analysed via SEM-EDX (Carl Zeiss, Germany; Model: Merlin Compact) for qualitative assessment and quantitative analysis of calcium and phosphorous. The data were analysed for multiple group comparison using IBM SPSS version 20 with one-way ANOVA followed by a paired *t*-test.

Results: Calcium/Phosphorous ratio of all experimental groups; Group 1($1.92 \pm .17$), Group 2 ($1.98 \pm .16$), Group 3 ($1.81 \pm .03$), Group 4 ($1.75 \pm .08$) was statistically different ($p < 0.0005$) from Control; while there was no difference between Group I and Group II ($p = 0.33$).

Conclusion: All experimental groups showed comparable remineralising potential. Even though no statistically significant difference is seen between Group I and Group II, after correlating with surface analysis it was concluded that Group II showed the greatest remineralising potential.

1. Introduction

Dental health remains a pivotal aspect of overall well-being and affects both the physiological and the psychological dimensions of an individual's life.

The oral cavity is a balanced biome, and any imbalance in physiological processes leads to a diseased state. Like every system in the body, turnover continuously occurs even in the oral cavity. The loss of minerals by the acidic environment of the oral cavity is compensated for by buffering action and reinstating minerals with saliva.[1]

Dental caries is a complex multifactorial disease characterized by demineralisation of dental hard tissue (enamel, dentine, and cementum) in deciduous and permanent teeth. If managed appropriately, dental caries is a preventable and a reversible disease. It is a complex and dynamic process in which a multitude of factors influence and initiate disease progression.[2]

The field of dentistry is shifting towards preventive and conservative approaches, with a growing emphasis on remineralisation as a key strategy for tooth preservation and restoration. Remineralisation is a natural process of restoring essential minerals to teeth enamel and dentin, is promising for mitigating demineralisation, decay, and dental erosion. The application of fluoride, which promotes remineralisation, is crucial in remineralising systems, as it increases the strength of the hydroxyapatite lattice structure.[3]

Casein Phospho Protein-Amorphous Calcium Phosphate (CPP-ACP) has demonstrated superior properties in situ in terms of anticariogenic activity, significantly increasing the levels of calcium and phosphate ions in supragingival plaques and promoting the remineralisation of enamel subsurface lesions.[4] The synergistic effect of CPP-ACP and fluoride (G C Tooth Mousse, Tokyo, Japan) in reducing caries is attributed to the formation of CPP-stabilized amorphous calcium fluoride phosphate, resulting in increased incorporation of fluoride ions into the plaque, together with elevated concentrations of bioavailable calcium and phosphate ions.[5]

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Calcium phospho silicate is a Bio-active glass (BAG) that was originally developed as a bone regenerative material in a class of highly biocompatible materials.[6] These materials are reactive when exposed to body fluids and deposit hydroxycarbonate apatite, a mineral that is chemically similar to natural tooth minerals.[7]

The combination of fluoride with functionalized tricalcium phosphate (f-TCP) not only provides greater remineralisation in terms of microhardness and fluoride uptake but also decreases the dose of fluoride required to achieve the same degree of remineralisation. ClinPro Tooth Crème (3 M ESPE, USA) is a .21 % w/w sodium fluoride (NaF) anti-caries dentifrice containing 950 ppm fluoride and f-TCP.[3]

Self-assembling peptide (SAP)11-4 is a rationally designed peptide, the monomers of which undergo well-characterized self-assembly into biocompatible fibrillar scaffolds in response to specific environmental triggers that mimic the enamel matrix. Around this matrix, enamel crystals are formed from calcium phosphate in the saliva. Curodont Protect (Credentis, Switzerland) is a combination of fluoride, calcium phosphate, and protein molecules.[8]

The advent of biomimetic agents has revolutionized remineralisation. However, there is a gap in the literature regarding the elemental and structural effect of these agents in comparison with fluoride-based systems. Therefore, we decided to analyze these products' effects after 21 days of treatment as directed by the manufacturer of the agents and evaluate the elemental disparities. There are still uncertainties and errors concerning their behaviour, timing, and manner of application. Despite the abundance of remineralising agents, it is critical to research novel tactics and compare various agents in order to enhance the remineralisation process. There is scope for more research in the aspect of remineralisation to understand the processes better and their biological and physiologic influences. The closer to our biological status is, the better the treatment outcomes.

Prospective caries studies entail measuring minor changes in the mineral content of teeth. SEM provides topographical images and is used to assess surface changes in the enamel. EDX quantifies many elements, including Ca, P, F, O, Mg, and Na, in terms of both atomic and weight percentages.[9,10] Thus, the present study aimed to analyze and compare the remineralising effectiveness of four different commercially available agents on artificial human enamel lesions after 21 days of treatment, by means of SEM combined with EDX.

2. Materials and methods

The study was approved by the Institutional Ethical Committee (AU/DAA/06/2022/FA105). This experimental study was conducted in the Department of Conservative Dentistry and Endodontics.

2.1. Sample selection

A total of seventy-five (75) upper and lower premolars, which were extracted for orthodontic therapeutic purposes in the Department of Oral and Maxillofacial Surgery, were included in this study. Sample size estimation was done by using GPower software (version 3.0). Sample size was estimated for F tests - ANOVA: Repeated measures, within factors, for 4 groups was chosen. A minimum total sample size of 48 (12 per group) was found to be sufficient for an alpha of .05, power of 80 %, .50 as effect size (assessed for difference in Mean Ca (%) in EDX, from similar articles).

2.2. Sample preparation

The selected teeth were thoroughly cleaned, and each tooth crown was embedded in resin, with the buccal surface facing upwards, exposed, and parallel to the horizontal plane. The buccal surface was polished using pumice slurry. A 3 mm × 3 mm window of exposed enamel was created in the middle of the sample surface via adhesive tape, and the sample was rendered resistant to acid attack by applying a uniform coat of nail varnish around it. Once the samples were adequately dried, the adhesive tape was removed, resulting in a rectangular area on the enamel surface.

2.3. Preparation of the demineralising solution

The demineralising solution was made by mixing 2.2 mM(Milli Molar) calcium chloride, 2.2 mM sodium phosphate, and .05 M (Molar) lactic acid and adjusting it to 1500 ml (milli-litre) with distilled water; the pH was adjusted with 1 M sodium hydroxide to 4.5.

2.4. Demineralising the samples

The samples were immersed in demineralising solution for 96 h to create artificial lesions in the enamel. The demineralized samples were checked via SEM-EDX. The teeth were then randomly divided.

1. Control: These samples were not subjected to any treatment post demineralisation and were stored in distilled water at 37 °C to avoid any changes due to environmental conditions.
2. Group I: CPP-ACP (GC Tooth Mousse, Recaldent, Tokyo Japan).
3. Group II: SAP 11-4 (Curodont Protect, Credentis, Switzerland).
4. Group III: f-TCP (ClinPro, 3 M, USA).
5. Group IV: BAG (Vantej, Dr. Reddy's, India).

2.5. pH cycling model

A pH cycling model provided by Featherstone et al.,[8] was used to simulate the changes occurring in the oral cavity. The remineralising pastes were applied with applicator tips and left on the tooth surface for 2 min, after which the samples were washed with distilled water. The samples were individually immersed in 20 ml of demineralising solution (pH 4.4) for a period of 6 h and washed with distilled water. This was followed by treatment of the samples again. All enamel samples were then individually immersed in 20 ml of remineralising solution (pH 7) for 17 h pH cycling was carried out for 21 days. The remineralising and demineralising solutions were replaced every 48 h and five days, respectively. After 21 days, the samples were analysed via SEM-EDX to determine the extent of remineralisation.

The data were analysed via the Statistical Package for Social Sciences (SPSS) version 21. The mean and standard deviation values of the Calcium: Phosphorous (Ca:P) ratios were calculated for the four experimental groups. The values were subjected to one-way ANOVA followed by paired *t*-test via SPSS software, and the p value $< .05$, was considered statistically significant.

3. Results

Scanning electron micrographs at 1500X displayed distinct morphologies of the enamel surface for each group (Figs. 1 and 2).

The results of the statistical analysis of the Ca/P ratio assessed using EDX analysis are shown in Graph I. The Ca/P ratio (Mean ± Standard Deviation) of the Group I- CPP-ACP (1.92 ± .17), Group II- SAP 11-4 (1.98 ± .16), Group III- f-TCP (1.81 ± .03), Group IV- BAG (1.75 ± .08) were statistically significant from Control ($p < 0.005$). Contrarily, there are no significant differences between Group I and Group II ($p = 0.337$) (see Table 1).

4. Discussion

Dental caries is a dynamic disease process in which equilibrium occurs between the pathogenic and preventive agents that cause demineralisation and remineralisation, respectively. Pathological factors include frequent sugar consumption, acidic foods and beverages, suppression of salivary function, and acidogenic bacteria, including antibacterial agents, salivary composition and flow rate, dietary fluoride

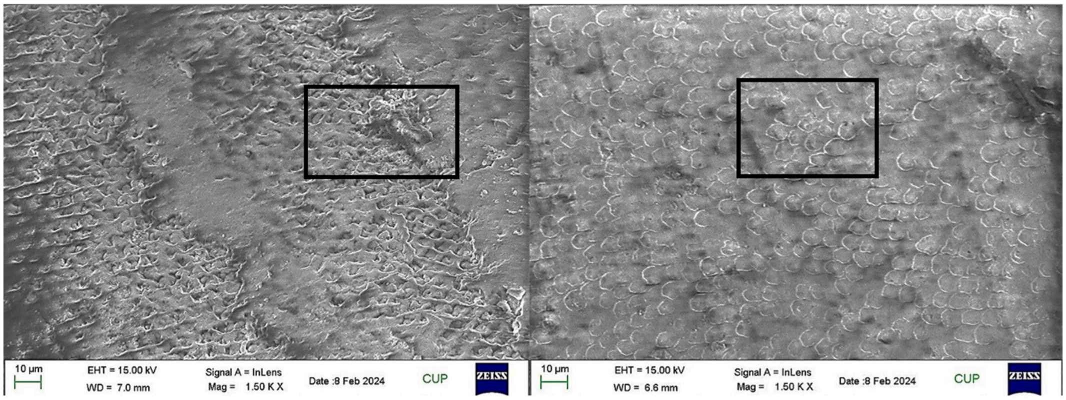

Fig. 1. SEM images of Group I and II. Group I(CPP-ACP) showed the presence of an irregular enamel surface, partial crystal recovery with remineralisation within the prismatic structure with intact interprismatic substance, Group II (SAP 11-4) showed superior enamel integrity with highly uniform deposition and hexagonal-comb-like matrix margins.

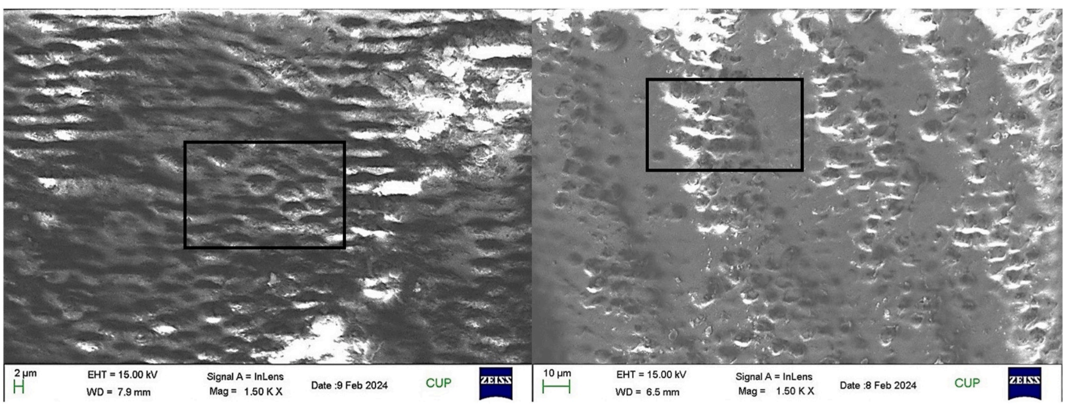

Fig. 2. SEM images of Groups III(f-TCP) and IV(BAG), Group III presented an irregular surface and partial crystal recovery within the prismatic structure. Group IV showed exposed enamel prisms, loss of interprismatic spaces and prism cores. The surface integrity was the least uniform and smooth.

Table 1
Paired *t*-test values showing comparison between all groups. (p-value < .05 = Significant*).

INTERGROUP	COMPARISON	Mean	Std. Deviation	p-value
GROUP I	With Group II	.06600	.25701	.337
	With Group III	.10800	.17490	.031*
	With Group IV	.16400	.17266	.002*
GROUP II	With Group III	.17400	.17808	.002*
	With Group IV	.23000	.23118	.002*
GROUP III	With Group IV	.05600	.07347	.010*

intake, and other extrinsic sources.[11] In this in vitro study, the effectiveness of four different agents on the enamel surface following exposure to an acidic environment was assessed and compared. The findings revealed that by treating the demineralized enamel surface, remineralising agents delivered ions that favoured subsurface mineral gain in several ways. To the best of our knowledge, this is the first time that these available remineralising vehicles, i.e., CPP-ACP, BAG, f-TCP and SAP 11-4, have been compared.

We used the SEM-EDX combined analytical technique for the qualitative investigation of the enamel surface morphology and the quantification of the Ca/P ratio as indications of enamel health.[12] Therefore, crystalline structure integrity and Ca/P values are important indicators of the agents' effects on the enamel remineralisation of several samples in the tested groups.

According to the results of our study, the demineralisation process caused a decrease in the Ca/P ratio values of the tooth (Control); this can be attributed to the acidic action and pH maintained under critical value of 5.5.[13] The four agents tested were compared to the Control group Ca/P ratios. The highest increase in the value means that the remineralising treatment achieved superior enamel recovery.

Based on our results, among the tested agents (Group I-IV); Group 2 was able to mineralise the tooth superiorly, followed by Group I,III and IV, upon 21 days of treatment. SEM observations of Group I and Group II at 1500X magnification showed surface with partial crystal recovery with re-establishment of the interprismatic enamel structure, leading to surface re-establishment with Group II showing far superior uniformity and smoothness. Soares et al.,[8] in their study demonstrated the significant effect of SAP 11-4 in remineralising the tooth surface in comparison to CPP-ACP. In accordance with Yu et al.,[14] our results suggest that the Tooth Mousse (Group I) has a considerable effect on increasing the remineralisation of enamel but visible surface topography difference was noted in our observation. Group III and Group IV also showed considerable increase in Ca/P ratio but inferior to the other groups. Upon SEM analysis the surfaces show an irregular morphology of the surface defined as a "honeycomb structure", with voids and numerous micropores due to the complete loss of prismatic structure integrity and minimal recovery was seen.

Fluoride is a widely used remineralisation treatment, while materials such as CPPs stabilize amorphous calcium phosphate and increase calcium and phosphate levels in dental plaques. Remineralising technologies, such as BAG and f-TCP, are being developed to mimic the physiological function of the enamel matrix. Advancements in science and research are moving towards a regenerative approach, mimicking enamel matrix function, with new biomimetic technologies such as SAPs

11-4.

Stephan[13] reported that enamel dissolves at pH 5.5, regardless of the solvent used. Remineralisation produces acid-resistant minerals by incorporating fluoride into new crystal surfaces, unlike enamel or dentin.[9] Remineralised enamel crystallites are more resistant to decalcification and maintain their original orientation.[15]

Several studies have used the pH cycling technique for periods ranging from 7 to 14 days.[8] Vitiello. F et al.,[12] evaluated the efficacy of remineralising agents and reported that after 7 days of treatment, only initial enamel structure reorganisation had taken place, and complete remineralisation was not obtained. Balakrishnan et al.,[5] investigated the remineralisation capability of toothpastes over 30 days and reported that the degree of remineralisation increased with increasing dose. In this investigation, a pH cycling procedure of 21 days was adopted because it is thought to be sufficient to evaluate the possible efficacy of the remineralising agents.[16]

This study uses artificial carious lesions on tooth surfaces to replicate naturally occurring caries characteristics, as they are reliable in research because of their sound subsurface structure.

Previous research has suggested brushing for 90 s, 2 min, or 4 min for optimal tooth paste effectiveness. Gopalyegani et al.,[17] reported that brushing for 2 min with bioactive glass and fluoride toothpaste increased the surface microhardness of enamel samples during each pH cycle. In this study, the test groups were subjected to tooth brushing for 2 min to simulate the normal recommended daily tooth brushing method.

Jayaranjan et al.,[18] reported that CPPs are essential for transporting ACP and localising the soluble calcium phosphate phase on the tooth surface because of their sticky nature, which can also increase calcium and phosphate levels in plaques by up to fivefold. The remineralisation capacity of CPP-ACPs has been demonstrated both in vitro and in vivo. This increased bioavailability of calcium and phosphorous could be the reason for the increased Ca/P weight in Group I.

Sindhura.[19] et al., demonstrated that SAP 11-4 is a low-viscosity monomeric peptide that stimulates enamel remineralisation in early carious lesions without extra calcium or phosphate. The matrix-mediated mineralisation theory of SAP 11-4 suggests that bioactive peptides synthesized from amino acids infiltrate subsurface lesions and form a three-dimensional fibrillar scaffold similar to the extracellular matrix. This serves as a nucleus for hydroxyapatite, which promotes tissue regeneration by drawing calcium and phosphate ions from the saliva, resulting in a higher Ca:P ratio. Kirkham et al.,[20] identified fibrils in twisted bundles, similar to collagen fibres, and reported that HA crystallized in vitro along these bundles, indicating nucleation sites. Schlee M et al.,[21] reported that P11-4 relies on natural remineralisation through saliva. The quality of saliva, including its calcium and phosphate concentrations, mineral content, pH, and flow rate, determines its effectiveness. This could explain why both CPP-ACP and SAP 11-4 presented statistically similar increases in Ca and P contents although scanning electron micrographs display an apparently superior intact structure recovery of the enamel treated with SAP 11-4.

Shah R et al.,[22] demonstrated that f-TCP resulted in 48.18 % tubular occlusion. This could be attributed to the 950 ppm fluoride concentration. There is no explanation given in previous literature regarding the poor remineralising capacity of f-TCP, but it can be thought of as a difference in mechanism, as it does not employ a sticky nature, which might lead to prolonged surface contact or matrix formation. Although the SEM results revealed that f-TCP has a remineralising capacity and surface deposition ability, the remineralising potential of f-TCP might not be as superior as that of SAP and CPP-ACP.

Previously, BAG was used to treat dentin hypersensitivity by blocking exposed dentinal tubules. Gillam et al.,[23] utilized regular Bio-glass and an experimental dentifrice in this way; BAG particles can partially block dentinal tubules but can be removed by washing. The poor remineralising capacity of BAG, can be attributed to its poor adhesion capacity as well as the mechanism of action of the agent.

The results of this study are similar to those of previous research conducted by Das.A(2020),[24] which concluded that CPP-ACP demonstrated a greater remineralisation capacity than BAG did. Another study by Balakrishnan A et al.,[5] concluded that CPP-ACP had better remineralising potential, and there was no statistically significant difference between the f-TCP and BAG groups.

The general agreement among dental professionals is that caries should be identified and managed as soon as possible, as nonsurgical methods can be used to reverse the condition during its earliest stages. Although fluoride has been proven to be a reliable and effective agent for preventing tooth decay, recent research has led to the discovery of numerous innovative compounds that can be used for targeted remineralisation therapy, offering additional and complementary benefits.[19]

The use of remineralising agents in dentistry has significant implications for clinical use and patient care. Remineralising agents help prevent the progression of early dental caries by promoting the repair and remineralisation of tooth enamel. This can lead to improved oral health outcomes and a reduced need for invasive treatments such as fillings or restorations. Additionally, remineralising agents can also be used in the management of tooth sensitivity. By strengthening and repairing the enamel, these agents can reduce sensitivity and improve patient comfort.

The results of the present study illustrate that the extent of remineralisation via these mechanisms is comparable. For a long time, CPP-ACP has remained the gold standard for remineralisation, but with the advent of peptide vehicles and biomimetic mechanisms, a gateway for newer and better modalities of remineralisation has emerged.

5. Conclusions

All the samples showed a comparable degree of remineralisation, but the greatest degree of remineralisation was observed in Group I and Group II. However, compared with the surface analysis, Group II showed a uniform surface with matrix formation, while the surface roughness and micropores were more prominent in the remaining test groups, leading to the conclusion that it not only remineralises but also creates superior surface integrity. Within the limitations of the present study, all the experimental groups presented variable degrees of remineralisation.

Limitations: The in vitro experimental setup closely mimics the oral biological environment, but multiple factors, such as salivary flow, salivary pH, dietary habits, lifestyle, etc., affect the rate and progress of remineralisation. Therefore, more in vivo studies and long-term clinical trials are needed to support the assisted remineralisation capacity of the tooth surface and to assess the benefits and possible adverse outcomes of the use of remineralising agents. Nonetheless, the offered data remains positive and, most significantly, illustrates the beneficial activity of certain remineralising products on dental enamel reorganisation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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