ORIGINAL RESEARCH

Evaluation of Remineralization Potential of Natural Substances on Artificially Induced Carious Lesions in Primary Teeth: An *In Vitro* Study

Pooja V Ravi[1], Rajakumar Sekar[2], Kavitha Ramar[3]

Abstract

Aim: To evaluate the remineralizing potential of natural substances on artificially induced caries lesions in primary teeth.

Materials and methods: A total of 50 primary molar teeth were selected and subjected to a demineralization process. Then samples were randomly divided into five groups for the remineralization process. Group I—colophony, group II—5% sodium fluoride (NaF) + colophony, group III—grape seed extract (GSE) + colophony, group IV—5% NaF + colophony + 10% peptide, and group V— GSE + colophony + 10% peptide. All the groups were subjected to remineralization using a brushing stimulator for 3,000 cycles. Assessment was done using Vickers hardness testing machine for evaluating the enamel surface microhardness (SMH) and scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) for evaluating the surface morphology and mineral content, before and after demineralization and after remineralization, the obtained data was analyzed statistically using IBM Statistical Package for the Social Sciences (SPSS) software version 25.

Results: The enamel microhardness results of this study revealed that remineralization of enamel was highest in group V (212.83 ± 64.416) and least in group II (137.83 ± 26.324) *p*-value of 0.038. SEM-EDX analysis revealed high calcium (Ca) and fluoride (F) content in groups II and IV, which was significant (*p*-value of 0.001) from other groups. Surface morphology evaluated with SEM revealed spherical globular agglomerates and scaffolding deposits on the enamel surface in groups III and V resembling the remineralization process.

Conclusion: Grape seed extract (GSE) with colophony and peptide is a superior natural alternative to NaF. Colophony also exhibited remineralizing potential in primary enamel.

Clinical significance: Natural remineralizing agents like GSE, colophony, and its combination serves as a potential alternative to overcome the toxic effect on long-term usage of F. These natural substances can be applicable in clinical conditions by incorporating toothpaste and varnish, which can be used as an alternative or adjuvant to the topical application of F.

Keywords: Colophony, Grape seed extract, Peptide, Primary teeth, Remineralization.

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Introduction

Dental caries is a pandemic disease which contributes to a 10 times higher percentage than other oral disease.[1] Dental plaque bacteria, dietary carbohydrates, and vulnerable teeth are thought to be a trio of essential elements which contribute to the emergence of dental caries. Caries formation is accelerated due to continuous alterations in pH and acidic changes arising from the fermentation of carbohydrates by microbial biofilm causing dissolution of hydroxyapatite (HAp) crystals, leaving a demineralized lesion.[2] Remineralization of the lesion is possible only when the resting pH is made stable, which can be facilitated by the addition of remineralization agents.[3] Thus, understanding the significance of biomaterials and minimally invasive approaches, such as remineralization therapies are important to reduce the formation of white spot lesion and minimize the caries burden in the pediatric population.[4]

Fluoride (F) has been utilized extensively in recent years to treat and prevent early caries lesions and its use is highly recommended by scientific, regulatory bodies.[4] Despite its advantages, excess F can be hazardous causing dental and skeletal fluorosis, hypersensitivity reactions, hypersalivation, gastric irritation, muscular spasm, and birth defects. Hence, F use is restricted in children below the age of 6 years to avoid its toxic effects, and alternative remineralizing agents were searched for.[5]

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Recent researches have shifted back to a time in identifying a natural product which could provide promising sources to treat oral disease. In ancient times, herbs were used in the prevention and curation of dental caries.[3] The key benefits of utilizing herbal substitutes are their accessibility, affordability, and less toxicity. The consumption of naturally occurring plant metabolite proanthocyanidins (PA) has been beneficial for oral health with remineralization and anticaries properties.[6] One such richest source of PA is GSE which acts by decreasing the frequency of enzymatic

breakdown of the collagen matrix, increasing the rate of collagen synthesis, and enhancing the conversion of insoluble collagen to soluble collagen during development, thereby increasing remineralization.[7]

However, both natural and synthetic remineralizing agents have the significant problem of being soluble in saliva. Additionally, aqueous solutions are more difficult to use in a clinical environment since they require more patient participation and do not give gradual, continuous release. To overcome this, a natural substance called colophony—a hydrophobic resin was incorporated in the remineralizing agent to prevent the dissolution of the remineralizing agent in saliva. Colophony, when triturated with ethanol and applied onto the tooth surface, resolidifies and forms a gel-like structure when the alcohol evaporates,[4] and the addition of peptides enhances remineralization by increasing the net mineral gain and inhibiting the loss of minerals.[8]

To the best of our knowledge, no studies have been conducted to assess the remineralization potential of GSE combined with colophony and peptide. The unique features of this study are the same oral environment is simulated by using human saliva and a brushing stimulator, all the remineralizing agents were made in gel consistency, which could be used in clinical applications and the use of pure extracts of natural agents can be an appropriate toxic free alternative for synthetic agents.

In this context, taking a biomimetic approach using natural agents into consideration, this *in vitro* study intends to assess the remineralization capacity of natural substances on experimentally created carious lesions in primary teeth.

Materials and Methods

A total of 50 noncarious primary human molars extracted due to physiological mobility or retained in the permanent dentition were collected. The study excluded teeth with enamel fractures or cracks, white spot lesions, any discernible discoloration, caries, teeth with hypoplastic lesions, developmental abnormalities, and teeth with pulp therapy. The sample size was calculated using G*Power software to test the difference between five independent groups using one-way analysis of variance (ANOVA) test. According to the findings, a total sample size of $n = 40$ with eight in each group was required to achieve a power = 0.80 with α error = 0.05. In order to prevent sampling errors, each group received two additional samples resulting in a total sample size $n = 50$ with 10 in each group.

The sample teeth were washed, and ultrasonic removal of debris was done. The teeth were analyzed under diagnodent, and scores >6 were excluded. The specimens were kept in normal saline until usage to avoid dehydration. Self-cure acrylic resin was used to embed each tooth with the buccal surface facing upwards. Acid resistant nail varnish was coated, leaving 4 × 4 mm window on the center of the buccal surface, sticking plaster on the exposed tooth surface to delineate the area to be studied and left aside to dry.[1] Then, the baseline enamel SMH, enamel surface morphology, and mineral content were evaluated using Vickers hardness test and SEM-EDX, respectively.

Samples were set up on the Shimadzu HMV-G31D microhardness tester for the Vickers hardness test after the acrylic mounting had been leveled to create a parallel plane. A nanoindent was created in the sample teeth using the diamond tip. The sample was positioned so that the indent would land on the section's enamel part when seen under a 40× microscope. Rhomboid indent length and depth were measured after a 100 gm force was applied for 15 seconds, and hardness values were determined using an average of three indentations on each sample.[1] The teeth from each group were fixed on carbon mounts and coated with a gold alloy coating using a procedure known as sputtering for SEM assessment. Thermo scientific scanning electron microscope (model—Apero-s) was used.

Demineralization Protocol

After the baseline evaluation, samples were subjected to a demineralization process for 48 hours at 37°C. The demineralizing solution contains 2.2 mM Ca chloride, 2.2 mM monosodium phosphate, and 0.05 mL lactic acid. Separately, each component was added to the deionized water with constant stirring. Each ingredient was given time to fully dissolve before the next one was added. A 50% NaOH solution was used to bring the pH of the solution down to 4.5 while keeping it at 37°C in the incubator.[1] After demineralization the samples were washed in distilled water allowed to dry and evaluated for microhardness, surface morphology, and mineral content.

After evaluation, the samples were divided into five groups ($n = 10$) as follows:

- Group I—colophony.
- Group II—5% NaF + colophony.
- Group III—grape seed extract + colophony.
- Group IV—5% NaF + colophony + 10% peptide.
- Group V—GSE + colophony + 10% peptide.

In this study, a natural hydrophobic resin-solid colophony was obtained (Generic mft pvt ltd), crushed to a fine powder, and thoroughly mixed until it was dissolved in ethanol at a concentration of 35% by weight.[4] The purest form of grape seeds was obtained and triturated to a powder form and macerated,[9] in the Department of Pharmacology. The measured 100 mg of GSE contained 97.8% of PA and 70 gm of gallic acid equivalent. The peptide concentration used in this study was based on previous studies.[4] The components of each group were mixed with colophony to obtain a gel form.

Remineralization Protocol

The samples in each group were treated with the allocated remineralizing agent using a brushing stimulator for 3,000 cycles with linear X, linear Y, and circular motion for every 1,000 cycles. The test agents were replenished with human saliva every 500 cycles. After being cleaned with distilled water, samples were put in human saliva and kept at room temperature. One medically healthy child's saliva was used to acquire human samples. Prior to sample collection, eating or drinking was not permitted for at least 2 hours. To produce saliva, the individual was encouraged to chew paraffin gum. Then, saliva was gathered and placed in a polystyrene tube to be frozen at 80°C. Saliva was completely defrosted in a water bath at room temperature before usage.[10] The remineralized enamel SMH are measured using the Vickers hardness test and enamel surface morphology and mineral content was evaluated using SEM-EDX.

Statistical Analysis

Data were presented as mean, standard deviation (SD), and paired *t*-tests were used to compare baseline and postdemineralization levels. The one-way ANOVA test was used to compare the two groups. ANOVA with the *post hoc* Tukey test was used to compare various data between groups. A *p*-value of 0.05 or less was regarded

as statistically significant. IBM SPSS Software version 25 was used for the analysis.

Results

SMH Evaluation

Before demineralization, the enamel SMH of all tooth samples ranged from 282 to 388 kg/mm^2, with a mean (SD) of 335.1753.439 kg/mm^2, while the values after demineralization ranged from 103 to 137 kg/mm^2, with a mean (SD) of 120.1717.25 kg/mm^2. The baseline enamel microhardness decreased significantly following the demineralization cycle ($p < 0.001$) and there was a noticeable distinction ($p < 0.047$) in enamel SMH after remineralization among the groups, as presented in Table 1. Multiple comparisons after remineralization between the groups are presented in Table 2. Group V had the highest postremineralization SMH values ($p < 0.005$), differing significantly from all groups except group III. There was no significant difference between groups III and V (i.e., groups V and III are equal $p \leq 1.00$). The least amount of SMH values was recorded in group II (137.83 ± 26.324). Figure 1 depicts the images of enamel SMH before and after demineralization and after remineralization among the groups.

Micromorphological Surface Assessment of Enamel under SEM (Qualitative Assessment)

- The SEM pictures of sound enamel revealed uniformly ordered enamel rods and crystals with distinct outlines (Fig. 2).
- The demineralized enamel showed a rough surface, disorganized, with a loss of structural characteristics which resembles the carious enamel (Fig. 3).
- Group I showed a blockage of the spaces between the rods, a virtually smooth surface with spherical deposits on it, and comparatively few signs of porosities (Fig. 4).
- Groups II and IV demonstrated the remineralized enamel with NaF. The demineralized portions were covered in many calcific fluorapatite deposits on the enamel surface (Figs 5 to 8).
- Groups III and V had amorphous clumps, spherical globular agglomerates, and scaffolding deposits on the enamel's surface that varied in size from spot to spot and appeared to be the beginning of the remineralization process (Figs 5 and 6).

SEM-EDX Analysis

The SEM-EDX analysis revealed a significant variation in Ca levels between the groups, with group II (30.49%), group IV (34.23%) having high Ca content after remineralization and was statistically significant from other groups ($p < 0.001$) as represented in Figure 9.

Fluoride (F) content was highest in group II (7.83%), followed by group IV (3.49%), and was statistically significant from other groups ($p < 0.001$), as represented in Figure 10.

Table 1: Comparison of the SMH between the groups. Numbers in the parenthesis indicate the minimum and maximum range

Groups (N = 50)	*Mean ± SD (minimum–maximum)*	*p-value*
Before demineralization (N)	335.17 ± 53.439 (282-388)	
After demineralization (N)	120.17 ± 17.725 (103–137)	$p < 0.001$*
Group I (10)	159.33 ± 35.172 (124–194)	
Group II (10)	137.83 ± 26.324 (111–163)	$p < 0.047$**
Group III (10)	210.00 ± 59.555 (151–269)	
Group IV (10)	170.33 ± 45.557 (125–215)	
Group V (10)	212.83 ± 64.416 (148–276)	

*One sample/paired *t*-test (*p*-value < 0.05); **, one way ANOVA test (*p*-value < 0.05) group I–V indicates after remineralization

Discussion

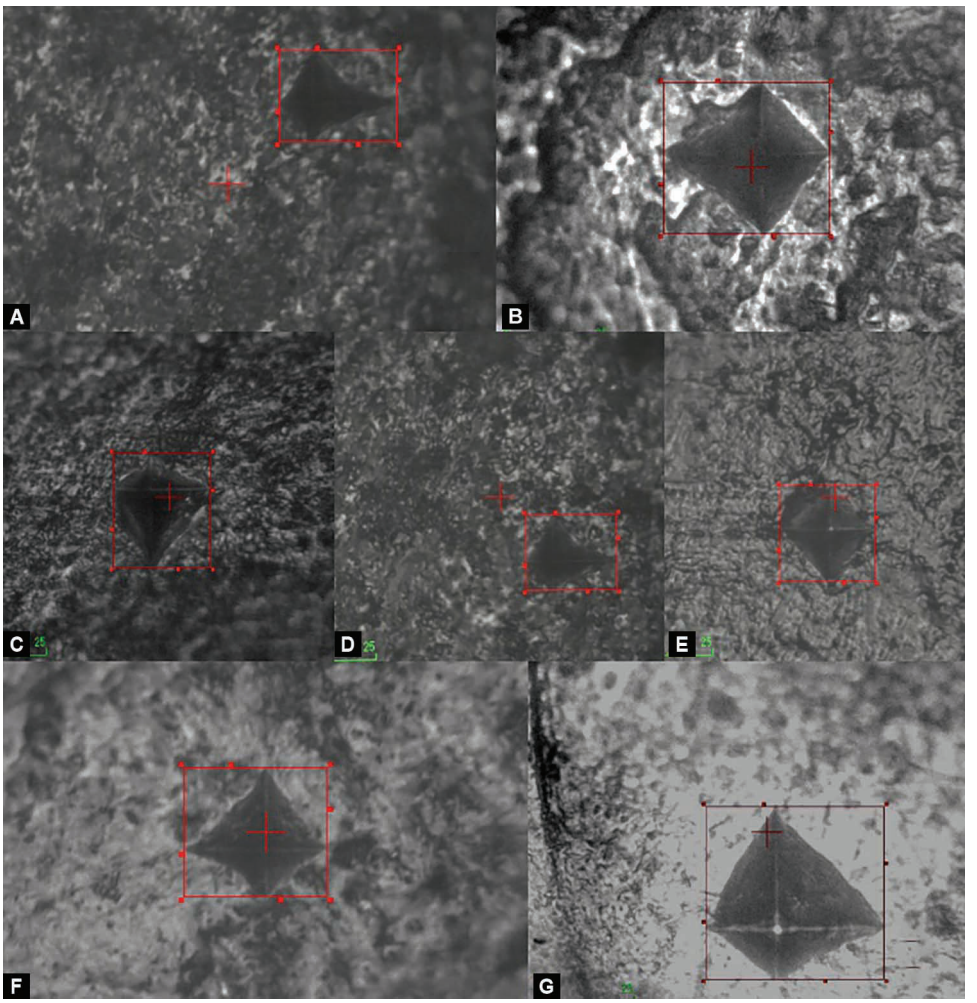
One of the most prevalent chronic disorders in children is dental caries which is brought on by an imbalance in the demineralization and remineralization cycles. This condition has a detrimental effect on the child's oral health as well as their self-esteem.[1] Remineralization therapies seek to replenish the lost mineral content by introducing Ca and phosphate into the demineralized pores, either from the saliva or external sources. F are well-known for their ability to prevent demineralization and encourage remineralization[4]. On applying F to the surface of enamel, hydroxide ions are replaced by F ions, forming a fluorapatite crystal structure which has less solubility and high acid resistance when exposed to demineralization by bacterial acids.[11]

Yet one of the major drawbacks of F is its ability to promote remineralization is limited by the availability of phosphate and Ca ions.[12] Further, F has efficient remineralization potential on smooth surface caries but has limited effect on pit and fissure caries.[4] Such limitations of F have prompted us to search for nonfluoridated natural alternatives for remineralization. Thus, the goal of the current *in vitro* investigation was to assess the ability of natural compounds to remineralize the experimentally produced caries lesions in primary teeth.

Table 2: Multiple comparisons of enamel SMH in between groups. ANOVA with *post hoc* Tukey test (*p*-value < 0.05)

Group (N = 10)	*Comparative group*	*Mean difference*	*p-value*
Group I	Group II	21.500	0.983**
	Group III	–50.667*	0.041*
	Group IV	–11.00	1.000**
	Group V	–53.500*	0.042*
Group II	Group I	–21.500	0.983"
	Group III	–72.167*	0.025*
	Group IV	–32.500*	0.040*
	Group V	–75.00*	0.038*
Group III	Group I	50.667*	0.041*
	Group II	72.167*	0.025*
	Group IV	39.667*	0.048*
	Group V	–2.833	1.000**
Group IV	Group I	11.00	1.000**
	Group II	32.500*	0.040*
	Group III	–39.667*	0.048*
	Group V	–42.500	0.045*
Group V	Group I	53.500*	0.042*
	Group II	75.00*	0.038*
	Group III	2.833	1.000**
	Group IV	42.500*	0.045*

*Significant *p*-value; **not significant



Figs 1A to G: Images of enamel surface microhardness: (A) Before demineralization; (B) After demineralization; (C) Group I; (D) Group II; (E) Group III; (F) Group IV; (G) Group V (C-G) representing after remineralization

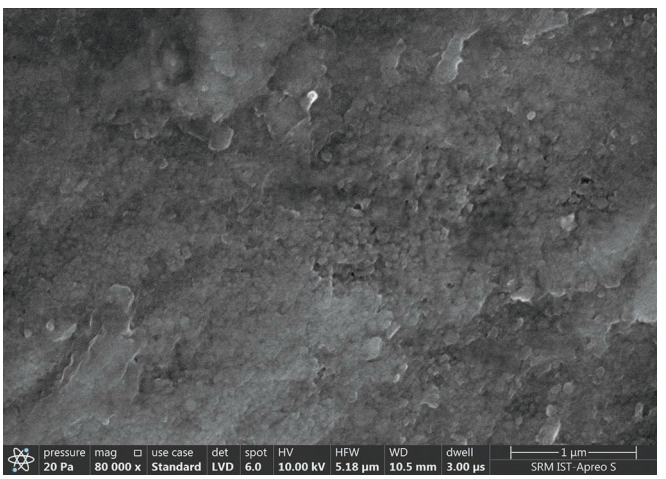


Fig. 2: SEM image of enamel before demineralization

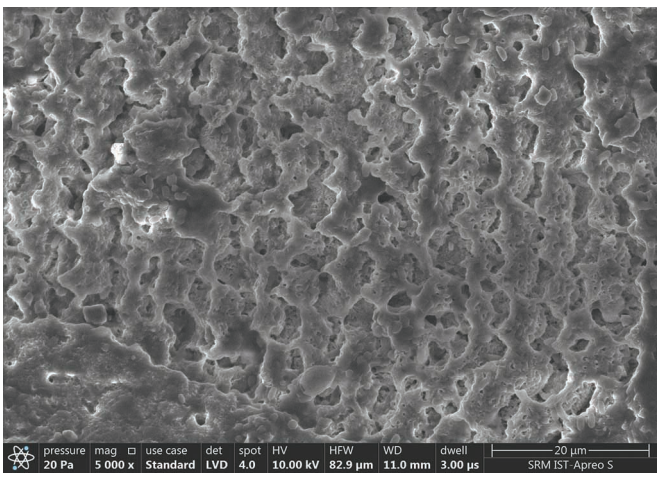


Fig. 3: SEM image of enamel after demineralization

The primary molar teeth were selected in this investigation for the development of artificial caries-like lesions because the primary teeth have soft, porous enamel, and a higher organic content than the permanent teeth, making them more vulnerable to caries than the permanent enamel.

Recently, GSE has been promoted for its advantageous remineralization, antimicrobial, and antioxidant capabilities. The major constituents of GSE are gallic acid and PA, where mineral deposition is facilitated by gallic acid, primarily on the surface layer and PA has an extremely high affinity to proline-rich

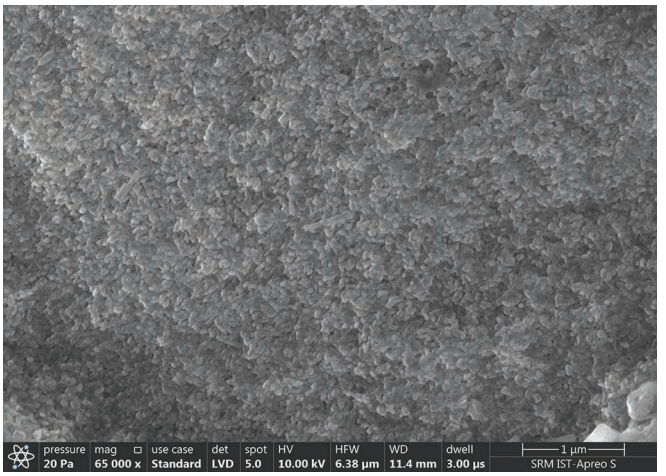


Fig. 4: SEM image of remineralized enamel in GROUP I

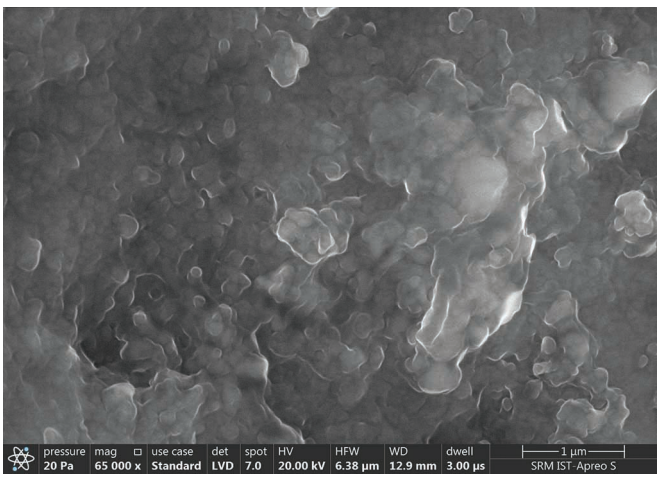


Fig. 5: SEM image of remineralized enamel in GROUP II

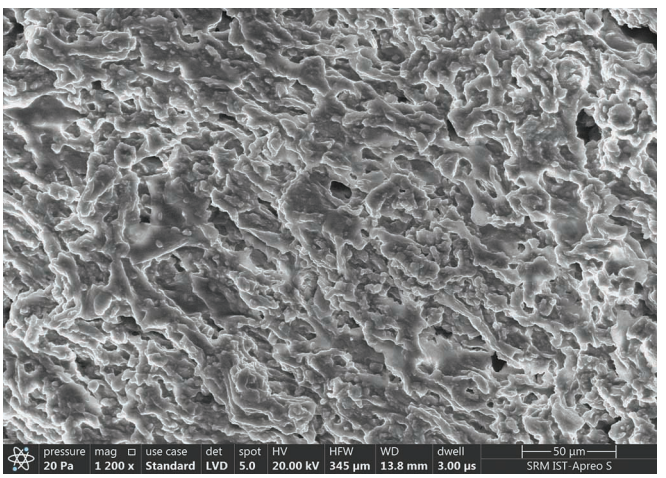


Fig. 6: SEM image of remineralized enamel in GROUP-III

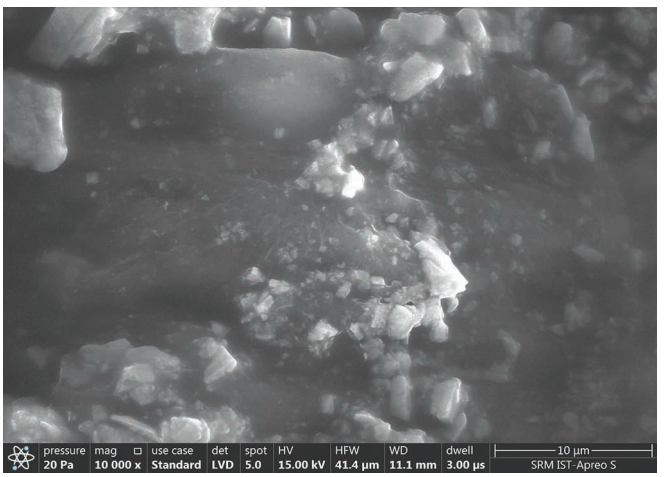


Fig. 7: SEM image of remineralized enamel in GROUP IV

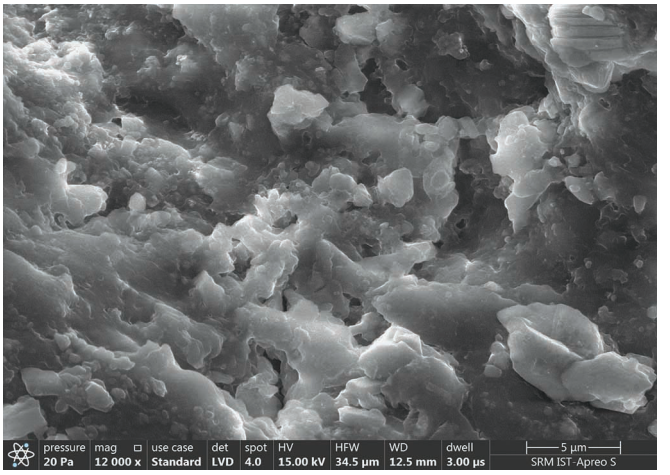


Fig. 8: SEM image of remineralized enamel in GROUP V

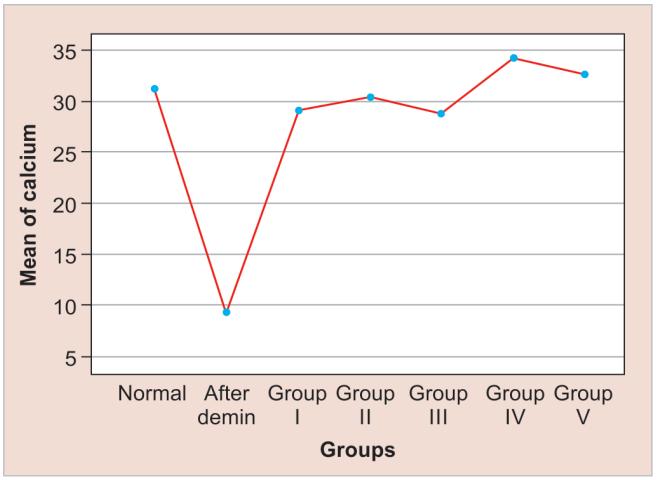


Fig. 9: Comparison of Ca levels between the groups

proteins like collagen and forms a proline–PA complex.[6,7] Açil et al.[13] demonstrated that type I collagen is detected in primary tooth enamel at minimal concentration, which was significantly lower as compared to that in dentin, despite the fact that traditionally mature dental enamel is thought to be free of collagen. Various studies done by Vijayapriyangha et al.,[3] Shiny Benjamin et al.,[14] have evaluated the remineralization potential of GSE but have not assessed the essentiality of type I collagen in primary enamel which can aid in positive effects of enamel remineralization. Hence in this *in vitro* study, a pure GSE was combined with hydrophilized collagen peptide to increase its remineralization capacity. However, aqueous solutions do not provide a favorable remineralization outcome as

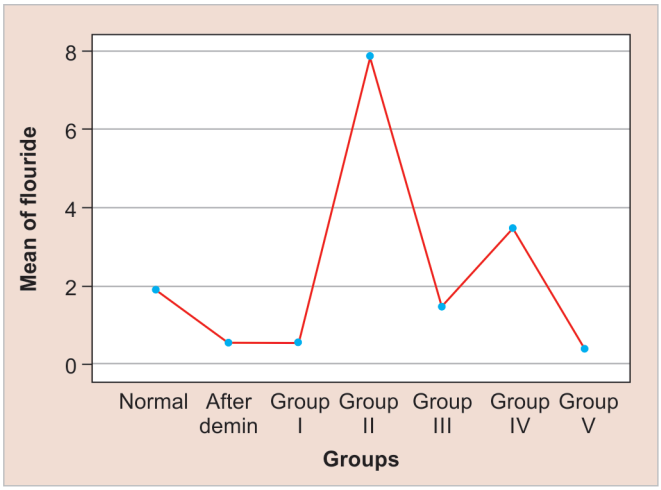


Fig. 10: Comparison of fluoride levels between the groups

it gets dissolved in saliva, so to overcome this, a hydrophobic rosin colophony was added to all the groups to bring it to gel consistency by which colophony component attaches for a longer amount of time on the surface of the enamel and constantly maintains the release of remineralizing agent due to its viscous nature, thereby enhancing the process of remineralization.[4]

The baseline values were recorded before the demineralization of all primary teeth, and the demineralization protocol was adopted as described by Gangwar et al. The samples were immersed in a demineralization solution for 48 hours at 37°C, after which postdemineralization SMH and SEM-EDX values were recorded, following which the five groups were divided randomly to evaluate the remineralizing potential.

The remineralization process has been carried out in numerous investigations across time periods ranging from 7 to 14 days.[1–3] Various dentifrices were tested for their ability to remineralize over the course of 30 days by Balakrishnan et al. They found that the dosage and exposure time were key factors in remineralization success.[15] Therefore, in the current study, in order to simulate the appropriate oral environment remineralization cycle was carried out using a brushing stimulator with 3,000 brushing cycles for each group which is a 3-month brushing period with 100 gm force and linear X, linear Y, and the circular motion was alternated for every 1,000 cycles and saliva was replenished for every 500 cycle.

Considering the importance of the surface layer in the advancement of caries, analyzing the changes taking place in this area is very important; thus, SMH measurement using Vickers hardness test, which is a relatively rapid, nondestructive, suitable, and simple technique for studying the enamel surface changes, deremineralization process, hence it was employed in this investigation.

In the current investigation, baseline Vickers SMH values were first determined by testing enamel samples. The reported average baseline value was 335.17 53.439. After the demineralization procedure, the samples were analyzed again, and after 48 hours, the mean value dropped to 120.17 17.725 (Table 1). Both values differed in a statistically significant way (p was 0.05) (Table 1). According to investigations by Mehta et al.,[16] Zhang et al.,[10] Lata et al.,[17] Shetty et al.,[18] and Neto et al.,[19] the SMH values were reduced. After the remineralization process, the SMH values were highest in group V, followed by group III, and the lowest was recorded in group II (Table 1). These results demonstrate that grape seed combined with peptide and colophony has a better capacity for remineralization than other groups. The addition of peptide compensates for the lack of external Ca and phosphate by promoting the precipitation of the Ca and phosphate that already exists, while the addition of colophony prolongs the retention of GSE on the enamel surface. Group I, which had blank colophony, also exhibited a mild remineralizing capacity. The NaF with colophony group had the least value in this study; this might confirm the factor that the action of NaF is dependent on the amount of Ca present in the saliva.[20]

The surface topographic changes seen in enamel were evaluated qualitatively using scanning electron microscopy. In the present research, the microphotographs obtained from SEM revealed different surface morphological patterns on usage of different remineralizing agents. The exposed enamel surface of the GSE showed a strong remineralizing impact (Figs 6 to 8). Few particles aggregated to form complexes and demonstrated several spherical deposits of calcific particles. Tang et al.[21] and Mirkarimi et al.,[7] reported similar results. Given that the majority of its structure is made up of inorganic material, this might be caused by the precipitation of minerals, especially in its interior enamel surface. It should be emphasized that the collagen peptides' ability to bind to the HAp surfaces is greatly aided by the terminal carboxyl and amine groups. The four distinct processes through which GSE interacts with proteins to create cross-links are covalent interaction, ionic interaction, hydrogen bonding interaction, and hydrophobic interaction,[7] which makes it a better remineralizing agent compared to NaF.

Since no pertinent studies have used energy-dispersive X-ray (EDX) analysis to provide quantitative compositional information in primary teeth, this analytical technique (EDX) was used in the current study in conjunction with SEM analysis to identify the elements in the deposited mineral particles and provide elemental identification, which revealed the baseline Ca level was 31.2% which dropped to 9.15% after demineralization, which is comparable to the research done by Lata et al.,[17] who found that initial enamel lesions with intact surfaces record a low mineral concentration at the surface layer when compared to sound enamel. This present study reported the highest Ca and F levels in NaF groups (group II and IV) after remineralization. Though the Ca and F levels were less in GSE groups (group III and V) they showed a remineralization potential because PA possesses a chelating mechanism with Ca ions, which promotes mineral deposition on the surface of collagen, and serves as a substrate for the production of apatite, and the addition of peptides promotes the production of HAp, mostly by serving as nucleation sites, and enhances remineralization without significantly changing the amounts of Ca.

According to the results of this *in vitro* investigation, GSE combined with peptide and colophony is a powerful remineralizing agent in comparison to NaF on simulated carious lesions in the enamel of primary teeth.

Conclusion

Based on our study result, it can be concluded that GSE combinations have a positive effect on primary enamel remineralization, and its effect is significantly found to be higher than NaF. Another herbal product, colophony, also showed an effective remineralization potential on primary enamel; these can be considered as an effective natural agent adjunct to other fluoridated and nonfluoridated synthetic remineralizing materials and a promising

noninvasive therapy for carious lesions in primary teeth. GSE combined with peptide and colophony can be incorporated into various other products like toothpaste, gel, and varnish and can be applicable in clinical conditions in place of or as an adjuvant to the topical application of F. The depth of demineralized subsurface lesion was not assessed in this study which will be evaluated in the upcoming study to obtain more promising results on the effects of the remineralization potential of GSE and colophony.

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