Original Article

Comparative evaluation of remineralization potential of four different remineralization agents on human enamel: An *in vitro* study

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Abstract

Aim: The study aimed to assess the remineralizing potential of four different commercially available agents using a Scanning Electron Microscope (SEM), energy dispersive X-ray (EDX) analysis, and Vickers Microhardness (VMH) Test.

Materials and Methods: Forty-four specimens (n = 11 per group) were prepared from extracted teeth. A window of 6 mm × 4 mm was made on all the specimens that represented three zones, namely, sound enamel, demineralized enamel, and remineralized enamel. The zone for demineralized enamel was subjected to four different remineralizing agents; casein phosphopeptide-amorphous calcium phosphate fluoride (CPP-ACPF), tricalcium phosphate fluoride (TCP-F), calcium sucrose phosphate (CSP), and self-assembling peptide (P11-4). Remineralization (REM) was assessed using VMH; the structural changes were assessed using SEM that was analyzed using EDX analysis. The specimens were subjected to a newer regimen of demineralization. One-way ANOVA followed by *post hoc* Tukey test was used with a level of significance at $P \leq 0.05$.

Results: There were no significant differences in VMH between the groups for sound enamel ($P = 0.472$) and demineralized enamel ($P = 0.116$). VMH was statistically significantly more for P11-4 and the least for CPP-ACPF ($P = 0.011$). A *post hoc* analysis revealed higher VMH for P11-4 compared to CPP-ACPF ($P = 0.014$) and TCP-F ($P = 0.035$). SEM showed a homogeneous layer of minerals for all groups except CPP-ACPF. TCP-F reported a higher degree of REM, followed by P11-4 as assessed using EDX analysis.

Conclusion: Self-assembling peptide (P11-4) exhibited a higher degree of REM than other remineralizing agents followed by CSP.

Keywords: Caesin phosphopeptides; calcium sucrose phosphate; energy X-ray dispersive analysis; P11-4; saliva; tooth remineralization; tri calcium phosphate fluoride

INTRODUCTION

White spot lesions are the earliest clinical evidence of enamel demineralization (DEM) that is confined within a superficial enamel layer. Its structural integrity is maintained with no localized breakdown; however, if left untreated and with continuous loss of minerals, it can lead to cavitation.[1,2] The process of remineralization (REM) happens during near-neutral physiological pH. It involves the replacement of lost minerals such as calcium and phosphate ions during the early stages of DEM, resulting in much larger, newer acid-resistant hydroxyapatite crystals.[3,4] In addition, REM is also possible in all cases where there is no frank cavitation of enamel surfaces.[5]

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This cyclic process of DEM-REM is an ongoing process that is influenced by many factors such as pH of saliva.[6] Saliva buffers the plaque pH and provides a reservoir of minerals adjacent to the enamel. However, the process of REM with saliva is slow, insufficient to produce net mineral gain, and has little improvement in the esthetics and structural property of deeper lesions.[7,8]

Although fluoride remains the best agent for REM,[9,10] other calcium–phosphate-based agents in various formulations are also being used. Some of the commonly used agents such as casein phosphopeptide-amorphous calcium phosphate fluoride (CPP-ACPF; GC – Tooth Mousse Plus®),[11] tricalcium phosphate fluoride (TCP-F; 3M™ ClinPro™ crème 0.21% sodium fluoride anticavity toothpaste),[12] calcium sucrose phosphate (CSP) (Toothmin Toothpaste),[13] and self-assembling peptide P11-4 (CURODONT™ REPAIR, credentis AG)[14] are also capable of remineralizing initial DEM.

CPPs are bioactive agents that stabilize ACPF and form (CPP-ACPF) nanocomplexes that decrease DEM and promote REM by localizing ACPF by maintaining a reservoir of calcium, phosphate, and fluoride ions exactly where it is needed.[15,16] Tricalcium phosphate is a blend of beta-TCP, sodium lauryl sulfate, or fumaric acid. The resultant "functionalized" calcium and "free" phosphate increase the efficiency of fluoride in REM by making calcium, phosphate, and fluoride accessible to the tooth surface, enhancing mineral growth, and strengthening the tooth structure.[17] CSP contains 11% calcium, 9.5% organic phosphate, and 2.5% inorganic phosphate, which reduces hydroxyapatite's dissolution rate in acid buffers and decreases enamel DEM.[18,19] Self-assembling peptides (SAP11-4) are newer biomimetic agents that have a high affinity for calcium ions and aid the body's natural enamel REM process.[20]

Various techniques are employed to assess the DEM and REM of enamel. The present *in vitro* study was designed to comparatively evaluate the REM potential of four different remineralizing agents on artificially demineralized human enamel through surface microhardness (SMH), Scanning Electron Microscope (SEM) examination, and energy dispersive X-ray (EDX) analysis.

MATERIALS AND METHODS

The study was conducted in the Department of Conservative Dentistry and Endodontics, Mahe Institute of Dental Sciences, Mahe, Puducherry UT, from 2019 to 2022. The permission to conduct the study was obtained from the institutional review committee (MINDS/PG-ETHICAL/008/2019-20). Forty-four freshly extracted noncarious central incisor and third molars were collected (irrespective of the arch), cleaned free of calculus, debris, and soft tissue, and stored in a 10% formalin solution. The crown was sectioned 1 mm below the cementoenamel junction with a slow-speed diamond disc and intact enamel was diagnosed by stereomicroscopic examination. Each tooth crown was embedded in resin with a buccal surface facing upward, exposed, and parallel to the horizontal plane. The buccal surface was flattened and polished using 400–1200 grit abrasive paper sequentially. The samples were then divided into four groups ($n = 11$ in each group; four groups). Enamel SMH using Vickers Microhardness test was measured in each sample at the beginning of the study, after enamel demineralization, and at the end of the study post-REM. In addition, a few samples were randomly selected and observed with SEM and subjected to EDX.

Enamel surface microhardness test

A 6 mm × 4 mm window of adhesive tape was applied over the sample surface using 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 dried, the adhesive tape was removed, and a Vickers diamond indenter (Schimadzu™) was used at a load of 100 g for 10 s at three points 100 μm apart. A builtin microscope measured the diagonal length of the indentations and displayed the Vickers hardness number (VHN). An area of 2 mm × 4 mm was selected and coated with nail varnish to preserve the undemineralized enamel. The specimens were then immersed in 300 ml of demineralization solution (pH 4.4) for 96 h to induce white spot caries-like lesions. The solution contained 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, and 0.05M acetic acid, 1M KOH.[21] The solution was replaced with a fresh solution every 48 h. After 96 h, each specimen was washed with de-ionized water, air-dried, and microhardness of demineralized enamel was obtained for all the groups. The demineralized enamel of 2 mm × 4 mm was again coated with a layer of acid-resistant nail varnish before subjecting the specimen to remineralizing agents [Figure 1].

To mimic daily changes in the oral cavity, pH-cycling was applied for a duration of 4 weeks. The REM toothpaste was applied for 5 min for the last remaining demineralized area of 2 mm × 4 mm, and the samples are subjected to 3 h of DEM twice daily, with 2 h of REM between the periods of DEM.[22] The teeth were placed in 50 mL of artificial saliva for the rest of the day.[23] The artificial saliva used in the present study is composed of 2.2 g/L gastric mucin, 0.381 g/L sodium chloride, 0.213 g/L $CaCl_2.2H_2O$, 0.738 g/L potassium hydrogen phosphate, and 1.114 g/L potassium chloride. Finally, the pH was adjusted to 7.00 with 85% lactic acid at 37°C to adjust the Ph of artificial saliva.[24]

The REM procedure was done on the remaining 2 mm × 4 mm by applying remineralizing dentifrice on all samples specific to each group. Samples were grouped according to REM agents used in the present study as follows:

- Group I: CPP ACPF (GC Tooth Mousse Plus; GC Corporation)

- Group II: TCP F (ClinPro™ Tooth crème; 3M ESPE)
- Group III: CSP (Toothmin Toothpaste; Group Pharmaceutical Ltd)
- Group IV: P11-4 (Curodont™ Repair; Credentis AG).

Each of the remineralizing dentifrices was applied using a standardized method of application [Figure 1]. A pea-sized amount of remineralizing dentifrice was dispensed onto a cotton applicator, applied on the tooth surface, and left undisturbed for 5 min followed by rinsing using deionized water [Figure 1]. The remineralizing solution contained 1.5 mM $CaCl_2$, 0.9 mM NaH_2 PO_4, and 0.15M KCl with a pH of 7.[25] This application was done for all 28 days of the study. The microhardness of the enamel specimen after REM was again recorded.

The SEM examination was done on three randomly selected samples from each group to compare microscopic variations between the groups that were treated with different remineralizing agents using ×2000. These were compared with positive and negative controls. In addition to SEM, the specimens were subjected to EDX analysis to determine the amount of minerals in tooth specimens, since the degree of REM was assessed by the amount of calcium and potassium in treated specimens.

The data were statistically analyzed using SPSS for Windows (SPSS version 22.0, IBM Corp., Armonk, NY, USA). One-way ANOVA was used to compare the mean microhardness values between the groups for demineralized and remineralized enamel, followed by *post hoc* test. Unpaired *t*-test was used to compare mean microhardness values between baseline and demineralized enamel and between demineralized and remineralized enamel. The level of significance was set at $P < 0.05$.

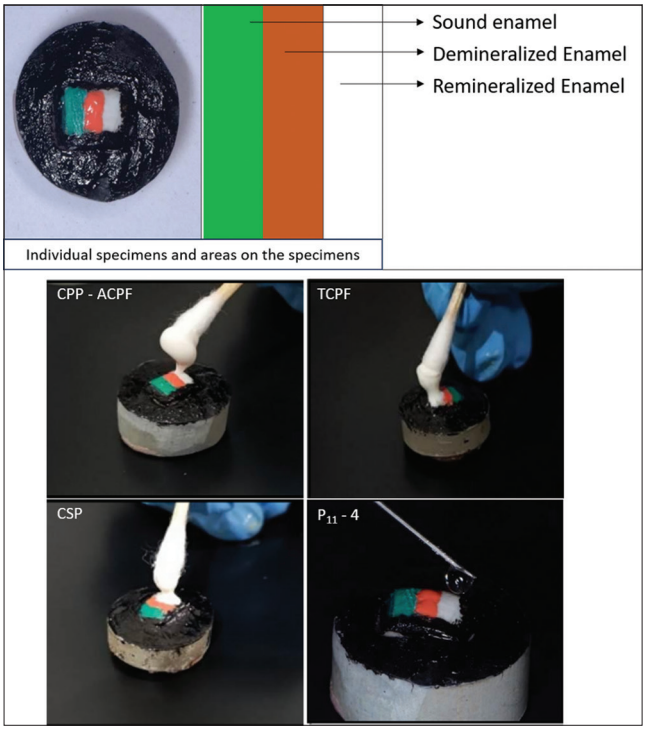


Figure 1: Final enamel specimen and application of remineralizing agent on test surface of specimens. CPP-ACPF: Casein phosphopeptide-amorphous calcium phosphate fluoride, CSP: Calcium sucrose phosphate

RESULTS

Surface microhardness tests

A one-way ANOVA test showed no statistically significant differences between normal enamel [P = 0.47, Graph 1 and Table 1] and demineralized enamel [P = 0.116, Graph 2 and Table 2]. However, statistically significant differences in mean microhardness values were found between the groups after REM. It was found that VHN values were significantly higher for P11-4 followed by CSP [Graph 3 and Table 3]. A *post hoc* test revealed significant

Table 1: Comparison of mean microhardness values of normal enamel

	n	Mean±SD	*F*	*P*
CPP - ACPF	11	278.8±34.1	0.85	0.472 (NS)
TCP F	11	302.4±38.2		
CSP	11	305.9±39.3		
P11-4	11	305.05±65.9		

NS using one-way ANOVA. SD: Standard deviation, CPP - ACPF: Casein phosphopeptide - amorphous calcium phosphate fluoride, TCP F: Phosphate fluoride, CSP: Calcium sucrose phosphate, NS: Not significant

Table 2: Comparison of mean microhardness values of demineralized enamel

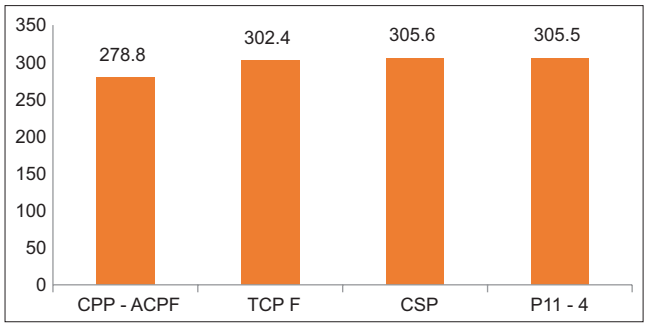
	n	Mean±SD	*F*	*P*
CPP - ACPF	11	88.8±18.3	2.1	0.116 (NS)
TCP F	11	92.5±10.7		
CSP	11	104.6±22.4		
P11-4	11	119.8±56.1		

NS using one-way ANOVA. SD: Standard deviation, CPP - ACPF: Casein phosphopeptide - amorphous calcium phosphate fluoride, TCP F: Phosphate fluoride, CSP: Calcium sucrose phosphate, NS: Not significant

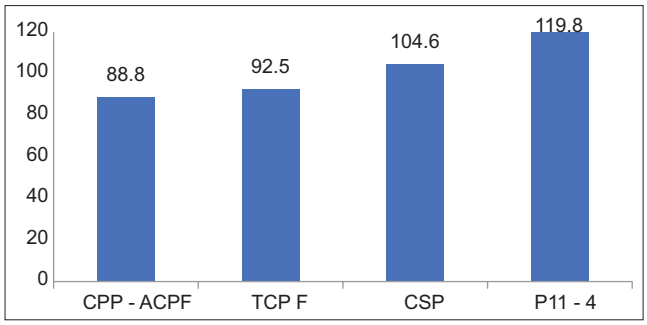
Table 3: Comparison of mean microhardness values of remineralized enamel

	n	Mean±SD	*F*	*P*
CPP - ACPF	11	121.76±16.2	4.2	0.011*
TCP F	11	126.58±12.8		
CSP	11	144.4±20.2		
P11-4	11	163.8±54.4		
Multiple comparison using ***post hoc*** test				*P*
CPP - ACPF versus TCP F				0.983
CPP - ACPF versus CSP				0.432
CPP - ACPF versus P11-4				0.014#
TCP F versus CSP				0.53
TCP F versus P11-4				0.035#
CSP versus P11-4				0.462

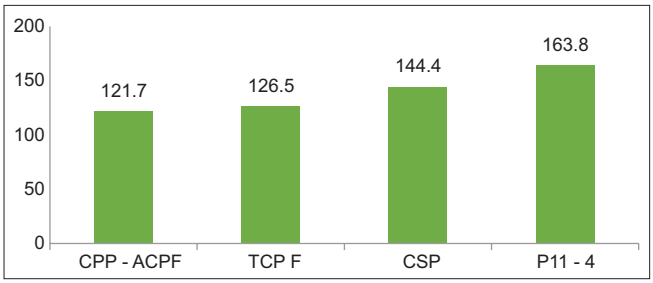
*Statistically significant at $P<0.05$ using one-way ANOVA, #Statistically significant at $P<0.05$ using Tukey's *post hoc* test. SD: Standard deviation, CPP - ACPF: Casein phosphopeptide - amorphous calcium phosphate fluoride, TCP F: Phosphate fluoride, CSP: Calcium sucrose phosphate



Graph 1: Mean microhardness values of normal enamel between the groups. CPP-ACPF: Casein phosphopeptide-amorphous calcium phosphate fluoride, CSP: Calcium sucrose phosphate, TCP-F: Tricalcium phosphate fluoride



Graph 2: Mean microhardness values of demineralized enamel between. CPP-ACPF: Casein phosphopeptide-amorphous calcium phosphate fluoride, CSP: Calcium sucrose phosphate, TCP-F: Tricalcium phosphate fluoride



Graph 3: Mean microhardness values of remineralized enamel. CPP-ACPF: Casein phosphopeptide-amorphous calcium phosphate fluoride, CSP: Calcium sucrose phosphate, TCP-F: Tricalcium phosphate fluoride

differences between P11-4 versus CPP-ACPF ($P = 0.014$) and between P11-4 versus TCP F ($P = 0.035$). There was no significant difference between P11-4 versus CSP [$P = 0.462$, Table 3].

SEM and energy dispersive X-ray analysis

An SEM of normal enamel revealed a characteristic fish-scale appearance with a smooth, intact surface. Following DEM, SEM of enamel showed rough, uneven, and increased porosities in addition to a minor honeycomb pattern in all groups. After 4 weeks of REM, the specimen subjected to CPP-ACPF still had multiple porosities and exhibited an irregular surface with the precipitation of minerals. For specimens subjected to other agents, an uneven yet homogeneous layer of minerals was visible that looked like obliterating the defect due to DEM and filled up the rods and interrod region. Furthermore, areas of calcified deposits consisting of irregularly shaped fluor-hydroxyapatite crystals were also evident, though areas of unfilled defects still persisted [Figure 2]. Table 4 lists the percentage of calcium and potassium content that was lost as a result of DEM and subsequently gained due to REM in different groups. It was observed that the percentage of REM was higher for TCP (ClinPro Tooth Crème) and P11-4 (Curodont™) [Table 4].

DISCUSSION

Recent cariology research focuses on the application of remineralizing agents on demineralized areas of teeth that help maintain a supersaturated environment of ions, thereby filling the microspores and helping in stopping mineral loss. The present study evaluated the remineralizing potential of four commercially available agents on permanent enamel samples using the pH cycling model.

The present study was designed to be an *in vitro* study where enamel samples were exposed to pH cycle modeling that helped simulate complex dynamics of the oral environment. These enamel samples were polished using a fine grit to create a flat uniform surface that facilitated the assessment of microhardness using VHN. VHN and SEM were employed in the present study, given the importance of the surface layer in caries progression. Microhardness measurement is a relatively simple, rapid, and nondestructive method to evaluate surface enamel changes. Vickers indenter has proven to be more effective because of the conservative square shape and its ease in the detection of errors at EDJ.[26] SEM, on the other hand, aids in reading surface topographical changes seen on enamel caused by the mineral deposition.

In the present study, the overall surface hardness of sound enamel was in the range of 252.2–339, which was similar to the range of values obtained by del Pilar and Reyes-Gasga (VHN 268-375) and Thabitha *et al*. (VHN 263-345).[27,28] The induced DEM had a VHN that was significantly less than the VHN of sound enamel, similar to Thabitha *et al*. and Lata *et al*.[28,29] The REM of enamel after the application of four different remineralizing agents and post-pH cycling witnessed significant differences in VHN values between the groups. It was found that P11-4 had the highest VHN and CPP-ACPF had the lowest VHN.

The present study witnessed that self-assembling peptides are emerging remineralizing agents that are an attractive alternative for the repair of demineralized enamel. The REM potential of P11-4 has been established both by *in vitro* and *in vivo* studies.[20,30] Mohamed *et al*., in 2020, conducted a systematic review and reported that the application of P11-4 is associated with significant enamel regeneration on demineralized surfaces.[31] These agents undergo spontaneous assembling after being applied to demineralized enamel and form a fibrillar network on which minerals are deposited, leading to REM. In addition, Schlee *et al*. showed that the application of P11-4 also improved REM in deeper layers of enamel.[32] Kamal *et al*. reported that P11-4 used in combination with CPP-ACPF is associated with significantly higher REM;[33] however, in the present study, CPP-ACPF was used as a separate comparison group.

Although CPP-ACPF was a better alternative than CPP-ACP alone,[34] in the present study, we found that CPP-ACPF

Table 4: Calcium and potassium ions as assessed by energy dispersing X-ray analysis for sound enamel, demineralized enamel and remineralized enamel in atomic percentage

Ca K	Sound enamel (a)	Demineralized (b)	Remineralized (c)	Percentage difference between (a) and (c)
CPP - ACPF	19.48	15.41	17.41	20.8
TCP F	23.45	18.02	21.27	9.29
CSP	20.47	17.92	19.04	6.9
P_{11}-4	21.72	17.09	20.74	4.51

CPP – ACPF: Casein phosphopeptide - amorphous calcium phosphate fluoride, TCP F: Phosphate fluoride, CSP: Calcium sucrose phosphate

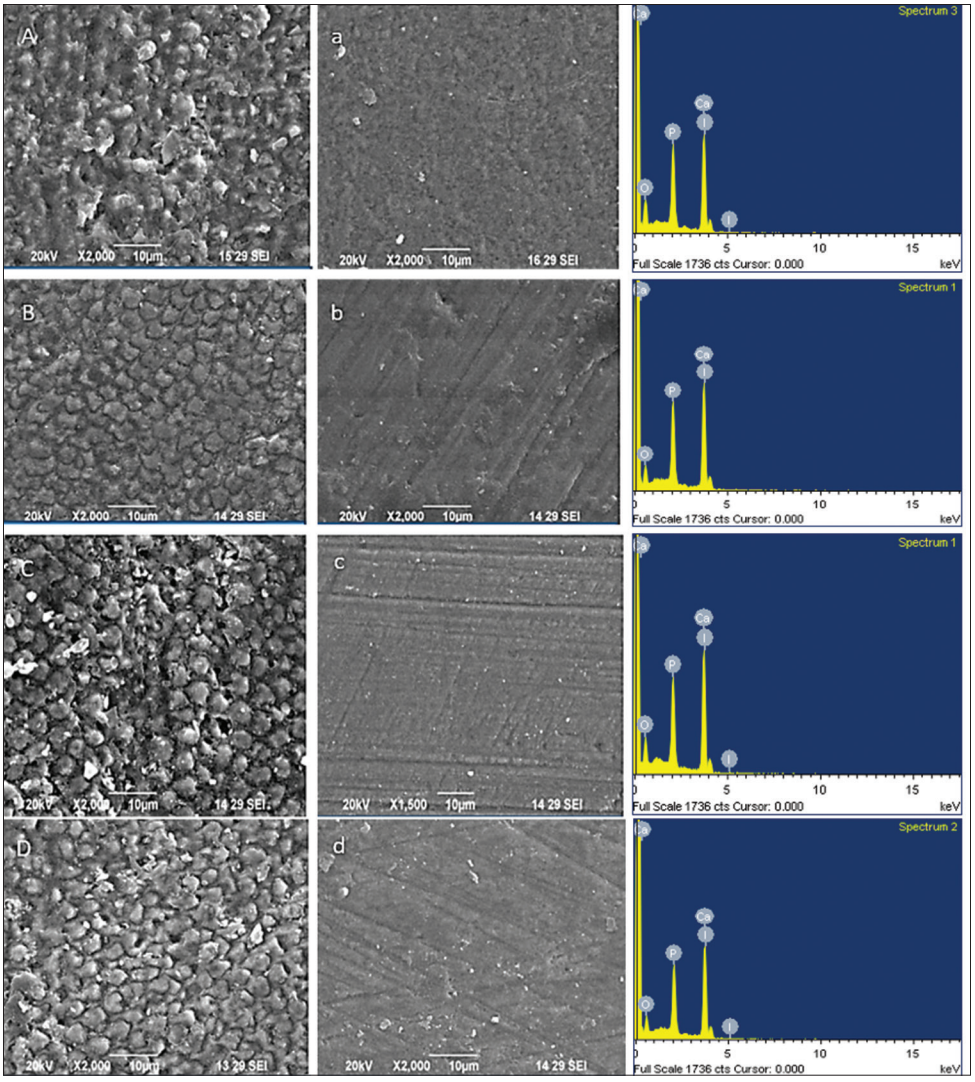


Figure 2: SEM of demineralized (uppercase) and remineralized (lowercase) enamel with energy dispersive X-ray analysis for casein phosphopeptide-amorphous calcium phosphate fluoride (A and a), Tricalcium Phosphate Fluoride (TCP-F) (B and b), calcium sucrose phosphate (C and c) and P11-4 (D and d)

had the least VHN values post-REM. This finding was in contrast to other reported studies where CPP-ACPF had high VHN values.[35] Lower VHN values were probably due to a difference in the exposure time of enamel specimens to a REM solution. The routine REM procedure involves subjecting the enamel specimens to 17 h of REM and 3 h of DEM. Instead, in the present study, we subjected these specimens to 3 h of DEM three times a day followed by 2 h of REM alternatively. This depicted the normal food intake, followed by a normal resting period in artificial saliva. This newer method of REM needs to be explored using well-designed *in vitro* studies before definitive recommendations can be established. Similar results were also reported by Shetty *et al*., who reported low levels of available fluoride, and Lata *et al*., who found that fluoride and CPP-ACP do not provide any additive REM potential when compared to fluorides.[29,35]

Although saliva is a major source of ions for remineralization, it fails to withstand extended periods of acidic challenges, and precisely for that reason, an exogenous supply of calcium and phosphate ions is provided that aids in remineralization.[36] TCP was considered a possible means of enhancing levels of calcium in plaque and saliva. Functional TCP has been introduced in tooth crème with 0.21% of sodium fluoride and marketed as ClinPro Tooth Crème.[37] In the present study, ClinPro Tooth Crème had lower VHN than P11-4 but had a higher VHN than CPP-ACPF, indicating better REM than CPP-ACPF. An *in vitro* study conducted by Bajaj *et al*. found that ClinPro Tooth Crème had the lowest REM effect when compared to CPP-ACP and hydroxyapatite,[25] and in yet another *in situ* study, Vanichvatana and Auychai found that REM of artificial caries was similar with ClinPro Tooth Crème and fluoride toothpaste.[38]

In the present study, CSP was found to have better VHN than CPP-ACPF and TCP F. CSP agent used in the present study is commercially available as Toothmin Tooth cream that is based on Anticay technology, which supplies both calcium and phosphate that maintains an alkaline pH. CSP acts by reducing the rate of dissolution of hydroxyapatite in acid buffer and decreasing enamel DEM.[19] A study conducted by Thabitha Rani *et al*. and Sargod *et al*. found that CSP had significantly higher mean SMH than CPP-ACP in addition to reducing the lesion depth.[28,39] A study by Veeramani *et al*. found CSP to be an agent of choice for primary dentition.[40]

In the present study, SEM of specimens treated with remineralizing agents revealed different findings that ranged from smooth intact surface for unmineralized enamel to interprismatic dissolution of enamel, porosity, prism irregularity, exposed underlying perikyamata, and deepened tomes process in demineralized enamel that was similar to findings by Thabitha Rani *et al*.[28] EDX analysis is a microanalytical technique that is used in conjunction with SEM for elemental analysis. The EDX X-ray detector measures the number of emitted X-rays versus their energy. A spectrum of energy versus relative counts of detected X-ray is obtained and evaluated for qualitative and quantitative determinations of the elements present in the specimen using a computer-based program.[41] EDX analysis revealed that specimen treated with TCP F had the highest percentage of calcium and potassium post-REM followed by P11-4. Interestingly, P11-4 had the highest VHN, followed by CSP and TCP F; however, the percentage difference between sound and remineralized enamel was found to be least in P11-4 and CSP, respectively. The finding of the present study was not in accordance with a study conducted by Shaik *et al*. who reported CPP-ACP with higher REM using EDX analysis.[42] In addition, Hegde *et al*. reported a higher degree of REM on the specimen using β-tricalcium phosphate than CPP-ACPF.[43] Furthermore, Shetty and Nekkati reported that P11-4 showed better remineralizing potential than a fluoride varnish.[44] The *in vitro* nature of the study was a limitation since CPP-ACPF works best in the presence of intraoral biofilm. Self-assembling peptides are the newer remineralizing agents that exhibited superior REM properties and further *in vitro* and *in vivo* studies are warranted for a detailed understanding of its mechanism. Furthermore, a novel Ph cycling has been used in the present study that was different from the usual Ph cycling method in the previous studies. This novel method might result in better REM of the enamel surface.

CONCLUSION

Within the limitations of the present study, it can be concluded that self-assembling peptide P11-4 exhibited the highest VHN after REM of enamel lesions. SEM with EDX analysis reported the least difference between sound enamel and remineralized enamel for P11-4, followed by CSP, TCP F, and CPP-ACPF, respectively.

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Conflicts of interest
There are no conflicts of interest.

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