ORIGINAL RESEARCH

Efficacy of Self-assembling Peptide P11-4 in Remineralizing *In Vitro* Caries-like Lesions in Primary Enamel Samples in Combination with Calcium Phosphate-based Remineralization Agents

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Abstract

Aims and background: The efficacy of self-assembling peptide P11-4 in combination with calcium-phosphate-based remineralization agents in remineralizing caries-like lesions in primary enamel was evaluated using a 21-day pH cycling experiment by Vickers microhardness [Vickers hardness number (VHN)] and scanning electron microscopy (SEM).

Materials and methods: A total of 120 primary enamel samples were made to undergo a demineralization cycle to produce caries-like lesions. They were divided into six groups, namely negative control (NC), positive control (P11-4), and four interventional groups in which each of the following calcium-phosphate-based agents were used in combination with P11-4—calcium sucrose phosphate (CSP), bioactive glass (BG), casein phosphopeptides, and casein phosphopeptides with fluoride. A 21-day pH cycling experiment was carried out with alternating demineralization and remineralization phases. The enamel samples were analyzed at baseline, post production of caries-like lesions, and post 21-day pH cycling using Vickers microhardness and SEM. Results were statistically analyzed using repeated measures of analysis of variance (ANOVA), keeping the level of significance at 0.05.

Results: Supplementing P11-4 with calcium-phosphate-based agents improved the surface hardness of the demineralized primary enamel samples, among which the fluoridated milk protein-based remineralization agent yielded a statistically significant improvement.

Conclusion: P11-4 promoted the regeneration of incipient caries-like lesions. However, there is added benefit when this peptide is used in combination with a fluoridated calcium-phosphate-based agent.

Clinical significance: This study would help the clinician compose an effective regimen for the patient to follow at home posttreatment with P11-4, in-office treatment.

Keywords: Calcium-phosphate, Caries-like lesions, Demineralization, pH cycling, Remineralization, Self-assembling peptides.

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Introduction

Literature-based studies have demonstrated the value of fluoride in preventing dental cavities and continue to assert that fluoride is still the gold standard for preventing caries lesions. Salivary remineralization, however, is also shown to be restricted to the tooth's outer 30 µm. The esthetic or structural aspects of the underlying lesion are not improved by this surface-only remineralization.[1] Fluorides have a notable penetrating effect on healthy enamel, but they are less effective on lesions that have developed into carious lesions.[1]

Due to their similarity to natural enamel, calcium phosphates were shown to be a viable biomimetic alternative in the field of remineralization. It is thought that calcium phosphate acts by penetrating the microscopic pores of developing carious lesions, where it serves as crystal nuclei in the remineralization process by persistently drawing significant volumes of calcium and phosphate ions into the lesion, thereby accelerating natural remineralization processes.[2] Manufacturers of the existing calcium-phosphate-based remineralization systems declare that these calcium phosphate systems help to supply calcium and phosphate ions for the remineralization process.[3]

However, since these conventional materials could not integrate with biological systems through a cellular pathway, a true regeneration strategy to regenerate hydroxyapatite crystals in the subsurface was required.[4]

Self-assembling peptides, P11-4 (CH3CO-Gln-Gln-Arg-Phe-Gln-Trp-Gln-Phe-Gln-Gln-GlnNH2), the recent advancement in the field of regenerative medicine, are used for regenerating tooth tissues on smooth surfaces.[5] This peptide, which is commercially available

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as Curodont Repair (Credentis AG, Switzerland), forms a three-dimensional (3D) matrix within demineralized carious lesion areas, enabling de novo hydroxyapatite crystal formation and facilitating the guided enamel regeneration of lost enamel structure.[6]

The effectiveness of this P11-4 depends on the individual's quantity and quality of saliva, particularly its mineral content, flow rate, and pH. This P11-4 genuinely depends on physiologic remineralization caused by saliva.[1]

Studies done in the past on self-assembling peptides have shown that enamel surfaces (ES) treated with them have improved mechanical characteristics than ES treated with fluoride supplementation[7] and also surface remineralization by formation of hydroxy apatite crystals[8] and, hence, proving to be a better remineralization agent when compared to casein phosphopeptide amorphous calcium phosphate (CPP-ACP), bioactive glass (BG), and fluoride-enhanced hydroxyapatite. Studies that used self-assembling peptides in combination with fluoride varnish (Duraphat) concluded that the biomimetic remineralization facilitated in this combination was not only safe but also effective and superior to the present clinical gold standard of fluoride therapy alone.[9]

However, no studies have reported the effect of P11-4, an in-office treatment method, in combination with home-care calcium-phosphate-based remineralizing agents.

The research question would be: does the usage of calcium-phosphate and fluoride-based home care agents influence the remineralizing behavior of self-assembling peptides?

The aim of this study was to determine the effects of self-assembling peptide scaffolds P11-4 alone and in combination with calcium-phosphate-based agents on the remineralization behavior of caries-like lesions of primary enamel under simulated intraoral conditions.

The alternate hypothesis proposed would be that supplementing the P11-4 treated primary enamel samples with calcium phosphates would have no additional effect.

Materials and Methods

Teeth Selection

Firstly, the institution granted ethical permission for this *in vitro* comparative study (No. 36/2020), and following that, the teeth specimens were collected. Sixty therapeutically extracted primary molars devoid of debris, stains, plaque, restorations, or cavitations, early caries lesions, hypoplastic lesions were meticulously screened and allotted for the study. Teeth with wasting disorders and developmental defects were excluded from participating in this research.

Sample Preparation

The 60 collected teeth were sectioned mesiodistally to yield 120 samples of primary enamel. After mounting the samples on the acrylic block, the outer 200 µm of surface enamel was removed sequentially using fine emery grits of 400, 800, 1000, and 2000 to provide a smooth and flat enamel surface. The samples were then polished using a pumice slurry. Following that, a 2 × 2 mm window was created on the surface by covering the remainder of the sample with nail varnish, which served as the area of interest (AOI).[10]

Baseline Readings

Vickers hardness testing equipment was used to determine the microhardness of the samples (Future Tech FM 800, Future Tech Corp, Japan). The Vickers diamond indenter was placed lightly on the test specimen using hydraulic damping with a force of 100 gm and a dwell duration of 10 seconds. This resulted in a rhomboidal depression with minimal sample damage. The diagonal length of the depression was measured at 50× magnification on the monitor. The surface microhardness was automatically calculated by the built-in software using the Vickers hardness number (VHN) formula, and the findings were recorded as VHN.

The surface topography was examined using scanning electron microscopy (SEM). Before SEM examination, the samples were air-dried for 24 hours on a Petri plate. After drying, the blocks were sputter-coated with gold palladium (BAL-TEC SCD 500 sputter coater) to a thickness of <50 nm. The samples were then examined and analyzed in a vacuum chamber of a scanning electron microscope (JEOL Scanning Electron Microscope, JEOL USA Inc., model JSM-IT300). The images were captured at a magnification of 1000×.

Creation of Caries-like Lesion

To develop *in vitro* caries-like lesions, the enamel samples were exposed to a demineralization cycle. The demineralization solution in this experiment was prepared according to ten Cate and Duijsters' methodology.[11] To minimize bias, the demineralization process occurred every 24 hours[12] using international caries detection and assessment system (ICDAS), and the cycle was terminated when a sample achieved an ICDAS score of 2.

Randomization and Grouping

Group I—Negative control (NC).
Group II—P11-4 + distilled water (P11-4).
Group III—P11-4 + calcium sucrose phosphate (P11-4 + CSP).
Group IV—P11-4 + bioactive glass (P11-4 +BG).
Group V—P11-4 + casein phosphopeptide (P11-4 +CPP-ACP).
Group VI—P11-4 + casein phosphopeptide fluoride [P11-4 + casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACPF)].

Application of Interventional Agent

The agent was supplied by the producer in the form of lyophilized powder. It was reconstituted by diluting with 50 µL of ordinary drinking water to obtain two to three drops of the product. The samples were treated within 10 minutes after reconstitution using a micropipette for approximately 5 minutes, before being returned to their artificial saliva storage solution.

pH Cycling

All the samples were exposed to pH cycling for 21 days. For 1 hour, the samples were immersed in 10 mL of freshly prepared demineralizing solution, divided into three 20-minute periods. Between each demineralization cycle, all samples were rinsed in deionized water and then immersed in 10 mL of freshly synthesized artificial saliva for 2 hours. The interventional agent was applied to the samples. Subsequently, the samples were incubated overnight in artificial saliva at 37°C in an incubator.[1] The solutions were freshly prepared and renewed everyday throughout the 21-day pH cycling experiment.

According to the guidelines obtained from the manufacturers, Elsenz (Group Pharmaceuticals, India) and Enafix (Group Pharmaceuticals, India) toothpaste are to be used twice daily, while GC Tooth Mousse (CPP-ACP) and GC Tooth Mousse Plus (CPP-ACPF) are recommended to be used once daily when

salivary secretion is higher. In this study, GC Tooth Mousse (CPP-ACP) and GC Tooth Mousse Plus (CPP-ACPF) were applied to the samples once daily, while brushing with Enafix and Elsenz toothpaste was conducted twice daily. pH cycling was carried out for 21 days.

A single operator brushed all the samples throughout the 21 days of pH cycling.

Postintervention Readings

Vickers hardness number values were measured on day 21 and tabulated. SEM images were taken at 1000× magnification.

Statistical Analysis

The obtained data was analyzed using Statistical Package for the Social Sciences (SPSS) PC version 24. Arithmetic mean and standard deviation (SD) were used for descriptive statistics. The significance of mean values was analyzed using repeated measures analysis of variance (ANOVA) and independent *t*-tests. The level of significance was set at 5%.

Results

Figure 1 shows changes in mean surface hardness within the groups in 21 days of pH cycling. It can be observed that except for NC, all other groups show statistically significant increased surface hardness values.

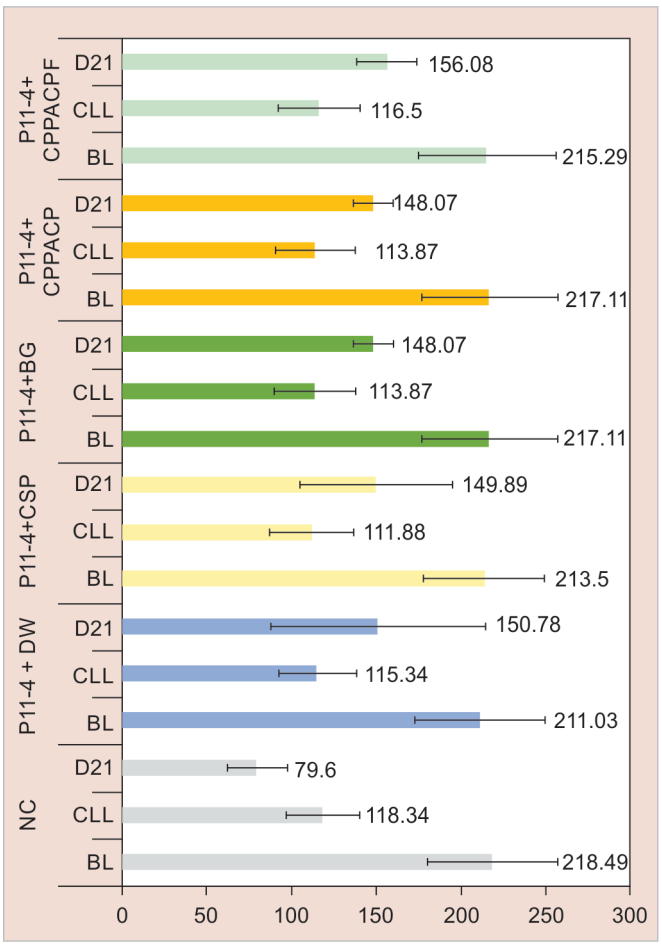

Fig. 1: Intragroup mean VHN among primary enamel samples across various time periods

In Table 1, it can be observed that compared to NC, all the test groups have gained surface hardness post 21 days of pH cycling. In contrast to the NC, which has lost its surface hardness, all groups except for P11-4 + BG showed statistically significant surface rehardening within the 21 days of pH cycling. Among them, P11-4 + CPP-ACPF showed the highest gain in mineral acquisition, followed by P11-4 + CSP, P11-4 alone, and then P11-4 + CPP-ACP.

In Table 2, it can be observed that all of the groups when compared against the NC, have shown statistically significant improvement in surface hardness. When the combination agents were plotted against the positive control (P11-4 + DW), no significant variation were noted.

Figures 2 to 9 are the SEM images taken at 1000× magnification. In Figure 2, the surface of enamel samples can be observed to be smooth and continuous which changes to uneven and porous post creation of caries-like lesions as observed in Figure 3. In Figure 4 showing the group of P11-4 + DW, the presence of scaffolds can be observed. In all other test groups, differences in surface topography can be observed compared to the image taken after the creation of caries-like lesions. The images from all test groups

Table 1: Intragroup mean difference of VHN among primary enamel samples across various time periods

		VHN	
Group	*Comparison between time periods*	*Mean difference*	*p*
NC	WSL vs pH cycling day 21	38.75	**0.001**
CR + DW	WSL vs pH cycling day 21	–35.44	**0.039**
CR + CSP	WSL vs pH cycling day 21	–38.01	**0.004**
CR + BG	WSL vs pH cycling day 21	–3.37	0.933
CR + CPP-ACP	WSL vs pH cycling day 21	–34.20	**0.001**
CRCPP + ACPF	WSL vs pH cycling day 21	–39.58	**0.001**

Bold values indicate significant level ($p < 0.05$)

Table 2: Intergroup mean difference of VHN among primary enamel samples across various time periods

	Day 21	
	VHN	
Groups	*Mean difference*	*p*
NC vs CR + DW	–71.18	**0.001**
NC vs CR + CSP-ACP	–70.29	**0.001**
NC vs CR + BG	–43.65	**0.038**
NC vs CR + CPP-ACP	–68.48	**0.001**
NC vs CR + CPP-ACPF	–76.49	**0.001**
CR + DW vs CR + CSP-ACP	0.89	1
CR + DW vs CR + BG	27.53	0.452
CR + DW vs CR + CPP-ACP	2.70	1
CR + DW vs CR + CPP-ACPF	–5.31	1
CR + CSP-ACP vs CR + BG	26.64	0.493
CR + CSP-ACP vs CR + CPP-ACP	1.81	1
CR + CSP-ACP vs CR + CPP-ACPF	–6.20	0.999
CR + BG vs CR + CPP-ACP	–24.83	0.578
CR + BG vs CR + CPP-ACPF	–32.84	0.24
CR + CPP-ACP vs CR + CPP-ACPF	–8.01	0.998

Bold values indicate significant level ($p < 0.05$)

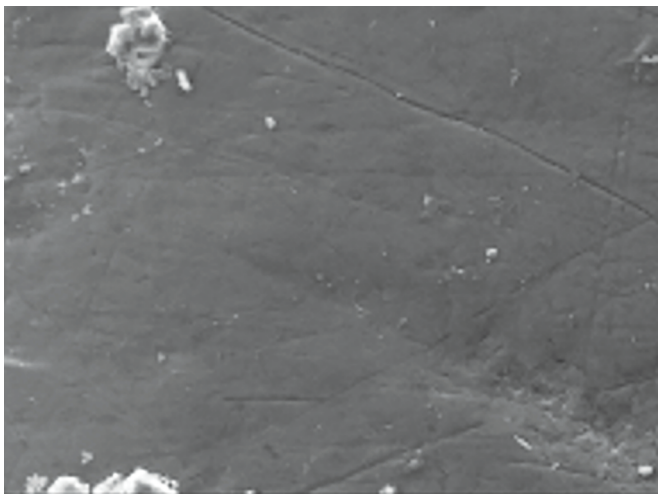

Fig. 2: Baseline

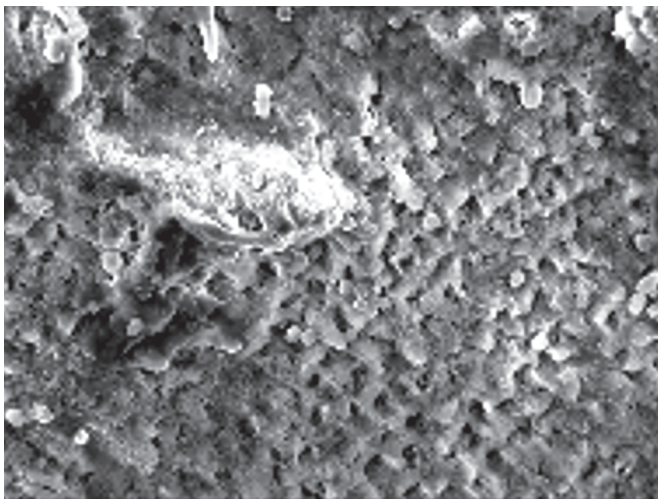

Fig. 5: P11-4 + DW

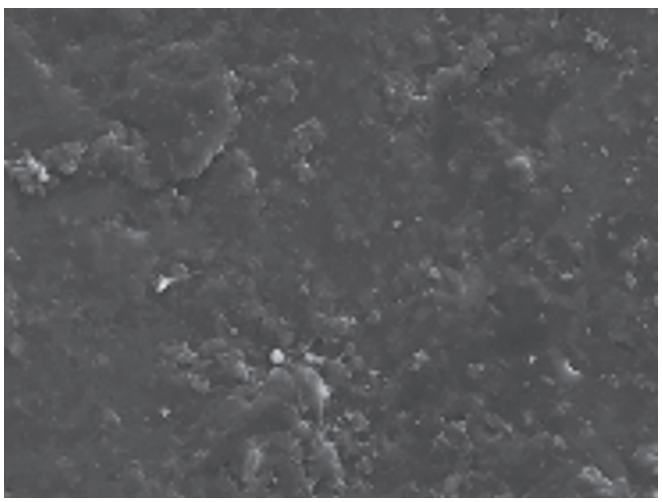

Fig. 3: Post production of caries-like lesions

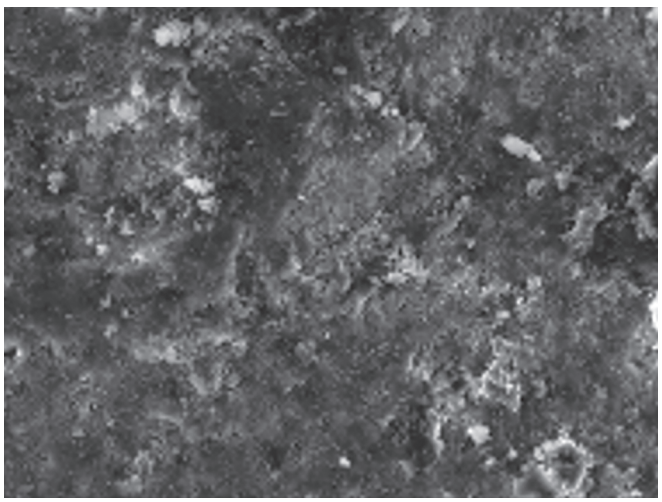

Fig. 6: P11-4 + CSP

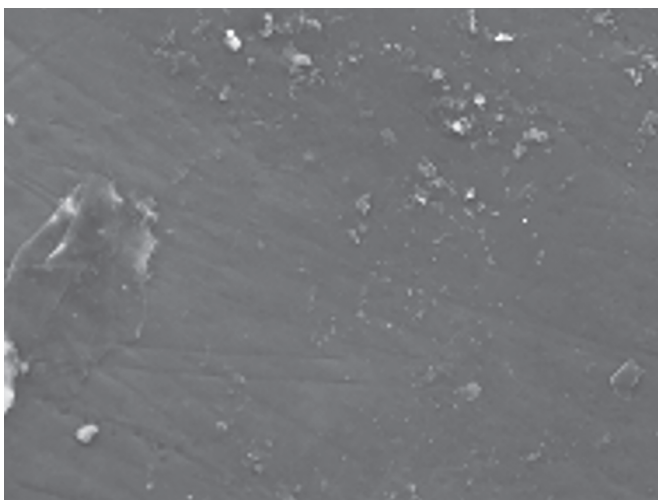

Fig. 4: Negative control

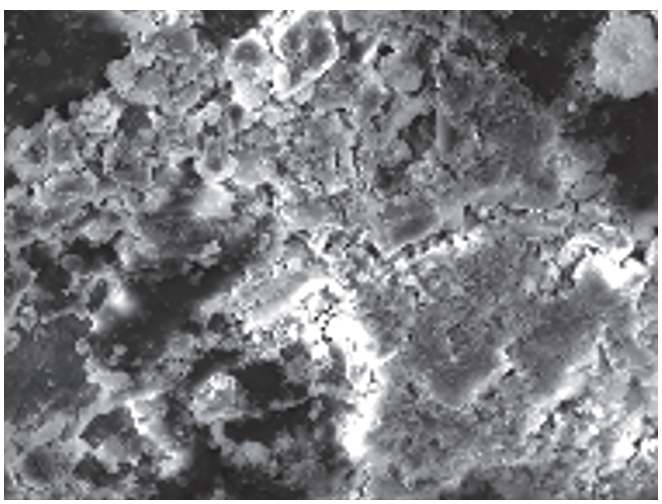

Fig. 7: P11-4 + BG

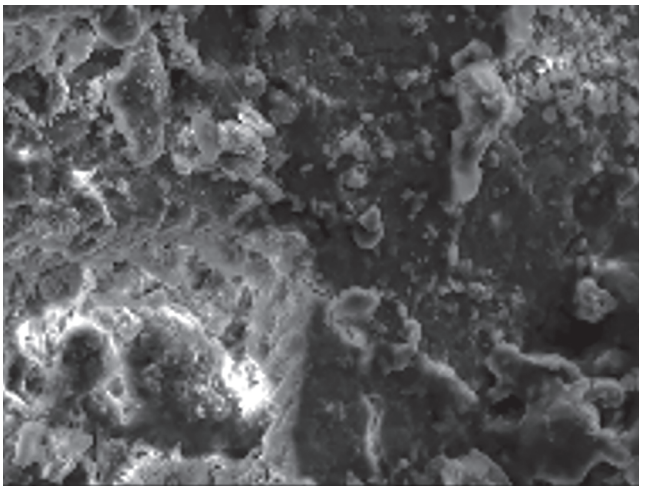

Fig. 8: P11-4 + CPP-ACP

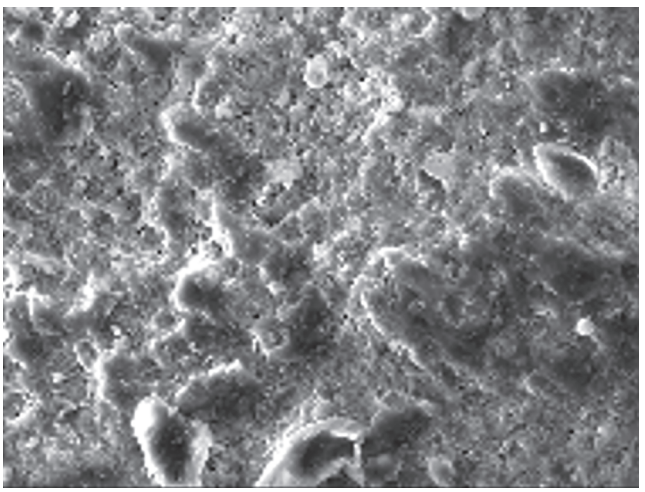

Fig. 9: P11-4 + CPP-ACPF

do not reveal significant porosities, which can be attributed to actual mineral deposition.

Discussion

A resolution on oral health approved by the World Health Assembly in 2021 calls for a shift from the traditional therapeutic strategy to a preventive one.

According to studies conducted in the past 10 years, several biomimetic systems might be used to mimic enamel-like microstructures, utilizing amelogenin-inspired polymers, peptides, and other organic components. This serves as an alternative strategy for the prevention and treatment of dental caries, which is highly desirable from both practical and scientific perspectives.[2]

P11-4 is a rationally designed peptide whose monomers self-assemble to form tapes and ribbons within seconds in response to specific environmental cues, and fibrils and fibers within the following 24 hours.[13] The SEM image (Fig. 4) clearly demonstrate the presence of scaffolds.

This biocompatible fibrillar scaffold accurately replicates the enamel matrix. This matrix is surrounded by enamel crystals formed from salivary calcium phosphate.[14]

Previous studies show that P11-4 has demonstrated excellent outcomes in *in vivo* and clinical studies as a biomimetic mineralization agent. *In vitro* studies using permanent enamel samples also reveal increased remineralization compared to casein phosphopeptide-amorphous calcium phosphate fluoride (CPP-ACFP) and sodium fluoride (NaF) on artificial caries lesions using DIAGNOdent and micro-computed tomography (μCT). Similar results were observed by Soares et al., where their study showed no significant difference between the P11-4 and CPP-ACFP groups. However, the study concluded that remineralization demonstrated by P11-4 was observed to be the highest, followed by CPP-ACFP, BG, and fluoride-enhanced hydroxyapatite gel.

The present study compared the in-office treatment option of using P11-4 alone to using the same agent in conjunction with home care calcium phosphate agents. This comparison aims to facilitate the development of a proper regimen that patients can follow in the comfort of their homes.

Among the most commonly used calcium phosphate agents available in the Indian market, two were chosen, including two fluoridated options (CPP-ACPF and BG).

The caries-like lesions were created using a chemical method of demineralization. According to Veeramani et al., they stated that in primary enamel samples, the white spot lesion begins to form within 48 hours. To avoid bias, the samples were monitored by two separate examiners and scored using the ICDAS method. The cycle was stopped when the lesion reached the clinical ICDAS 2 score.

There are various proposed pH cycling models for remineralization experiments. However, we chose the one proposed by Kirkham et al. in 2007, as this model closely resembles the intraoral environment with three cycles of 20 minutes of acid exposure every 24 hours.

Vickers microhardness testing was chosen because it provides a direct measure of mineral gain and correlates well with the amount of remineralization.

The results show that the use of calcium phosphate-releasing materials (CR) alone causes significant remineralization. This result is in par with the observations made by Özdemir et al. in 2022,[15] wherein they compared the remineralization potential P11-4 and other remineralization agents in primary ES.

When P11-4 was used in combination with milk protein-based remineralization agents, the results revealed statistically significant improvement in surface rehardening of caries, among which the fluoridated agent showed better results in primary enamel samples. This finding is consistent with a clinical trial that concluded that the gold standard of fluoride treatment alone is less effective than the biomineralization promoted by P11-4 in conjunction with fluoride.[9] There is indeed an added benefit when combining P11-4 with fluoride. In contrast, BG, which also contains approximately 950 ppm of fluoride, did not show as much improvement in surface hardness compared to CPP-ACPF.

It can be understood that the scaffold formed by P11-4 absorbs the minerals provided by the use of calcium phosphate agents, thereby increasing the amount of remineralization.

Overall, 100% reversal of carious lesions could not be achieved, which is a limitation of the study. Additionally, since the present study was conducted *in vitro*, further studies using *in vivo* setups are needed.

Conclusion

Within the limitations of the study, the following can be concluded. P11-4 promoted the regeneration of incipient caries-like lesions. Among the different agents used in the study, milk protein-based remineralization agents yielded better results. It can also be noted that there is an added benefit when this peptide is used in combination with fluoridated calcium phosphate-based agents.

Clinical Significance

This study would help the clinician to compose an effective regimen for the patient to follow at home posttreatment with P11-4, an in-office treatment.

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