Original Article

pISSN, eISSN 0125-5614
M Dent J 2025; 45 (1) : 21-32

Effect of self-assembling peptide with fluoride on remineralization of primary teeth: An *in vitro* study.

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Objectives: This study aimed to assess the remineralizing potential of a self-assembling peptide with fluoride (SAPF) on primary teeth compared to fluoride varnish and no treatment (control).
Materials and Methods: Thirty sound primary incisors were used, and surface microhardness (SMH) was measured before and after creating artificial enamel caries. The teeth were divided into three groups: SAPF, fluoride varnish (F), and control. After treatment and pH-cycling, SMH values were examined, and the percentage recovery of SMH (%SMHR) was calculated.
Results: SMH values after pH-cycling were significantly higher in the SAPF group (180.09±7.47 VHN) and F group (186.85±10.94) compared to the control group (117.45±8.17VHN) (p<0.001), but there were no significant differences between the SAPF and F groups (p=0.313). The %SMHR increased significantly in both SAPF (21.93±4.89%) and F group (25.75±10.14%) compared to the control group (-9.93±6.86%) (p<0.001).
Conclusions: The self-assembling peptide with fluoride demonstrated efficacy in remineralizing primary teeth comparable to fluoride varnish in vitro. This suggests its potential as an alternative treatment for dental caries.

Keywords: dental caries, enamel microhardness, primary teeth, self-assembling peptide, fluoride

How to cite: Udomsit A, **Prapansilp W**, Rirattanapong P. Effect of self-assembling peptide with fluoride on remineralization of primary teeth: An *in vitro* study. M Dent J 2025;45(1): 21-32.

Introduction

Dental caries remains a significant global health challenge, leading to discomfort, functional impairment, and aesthetic issues [1]. It is a dynamic condition characterized by cycles of demineralization and remineralization of tooth enamel, offering opportunities for preventive and regenerative interventions. The principle of minimal intervention dentistry focuses on arresting and reversing early carious lesions and promoting the regeneration of enamel subsurface structures [2, 3].

Fluoride is well-recognized for its remineralizing properties and plays a central role in the prevention of dental caries by enhancing the remineralization process at the tooth surface [4]. Despite the effectiveness of fluoride, there is an ongoing search for additional treatments that can enhance or mimic natural remineralization processes due to the limitations of fluoride alone in fully reversing the caries process and its potential risk of causing dental fluorosis at high concentrations [4].

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Received: 20 November 2024 **Revised:** 31 January 2025 **Accepted:** 8 February 2025

Among these additional treatments, self-assembling peptides (SAP), particularly the P11-4 peptide, have emerged as promising agents. SAPs mimic the natural proteins found in the enamel matrix, forming a scaffold that facilitates the nucleation and growth of hydroxyapatite crystals, which are essential for enamel regeneration [5]. When introduced into the oral environment, these peptides self-assemble into a three-dimensional matrix that integrates with saliva-derived calcium and phosphate ions, promoting the formation and repair of enamel structure [5, 6]. Using self-assembling peptides has demonstrated significant remineralization potential in both permanent and primary teeth [7, 8].

A previous study has demonstrated higher remineralization potential when self-assembling P11-4 peptides were followed by the application of fluoride varnish (2.26% fluoride) [8]. Nowadays, self-assembling P11-4 peptides containing 0.02% fluoride (SAPF) are available in the market. Despite the clinical interest in these peptides, there have been no studies on the remineralization potential of self-assembling P11-4 peptides with 0.02% fluoride (SAPF) since their introduction.

Primary teeth have thinner enamel and lower mineral content compared to permanent teeth, making them more susceptible to demineralization and caries progression [9]. While fluoride treatments are effective in primary teeth [4], there is an ongoing need for remineralization options that can address these specific structural vulnerabilities while minimizing the risk of fluorosis during this critical developmental period.

Therefore, this study aimed to evaluate the remineralizing potential of self-assembling peptides with fluoride on enamel microhardness of primary teeth. By focusing on SAPF, this research seeks to fill the gap in our understanding of how this particular formulation affects the microhardness of enamel, a critical factor in the resistance of teeth to caries. Through a detailed comparison, this study contributes to a nuanced understanding of SAPF alongside traditional fluoride varnish, enhancing our knowledge base for informed decision-making in dental care.

Materials and Methods

Setting and Design

This study was approved by the Ethics Committee of Mahidol University (COE.No.MU-DT/PY-IRB2022/DT031) to evaluate the remineralizing potential of a self-assembling peptide with fluoride (SAPF) on primary teeth, in comparison with fluoride varnish and a control group receiving no treatment. The experimental design and flow are shown in Figure 1.

Sample size calculation

Based on Kamal et al. (2020), sample size determination utilized one-way ANOVA, with a significance level (α) of 0.05 and a test power (1-β) of 0.9. To enhance the study's reliability and validity, ten teeth per group were selected, exceeding the adequate number of six teeth per group. This formed three groups: SAPF (Curodont Repair Plus™), fluoride varnish (F) (Duraphat® Varnish), and control (no treatment).

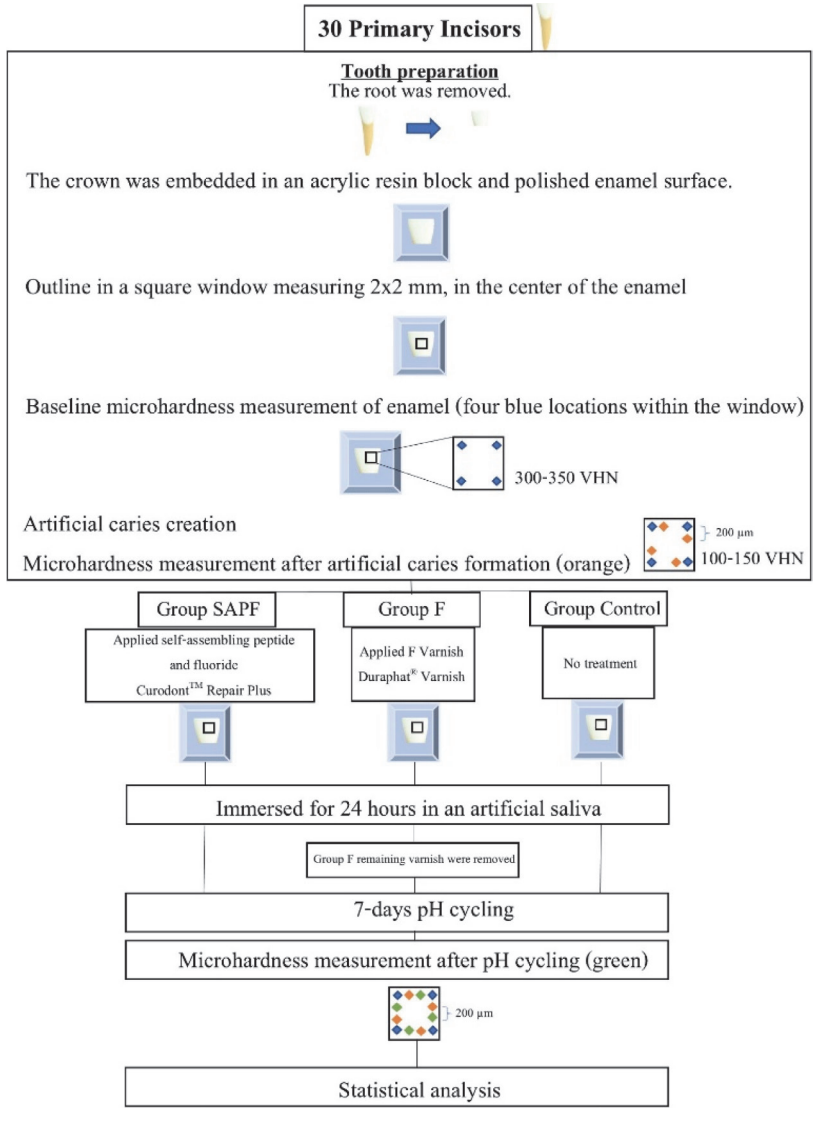

Figure 1 Representative scheme of methodology and experimental design

Specimens preparation

Thirty primary incisors were stored in normal saline at room temperature until used. Each tooth was cleaned and examined for any imperfections through visual inspection. The inclusion criteria were sound primary incisors without visible defects, no previous restorative treatment, free from cracks, caries, or white spot lesions, and stored in normal saline at room temperature. Criteria for exclusion were the presence of enamel surface abnormalities, cracks, caries, or white spot lesions, and restored teeth. Teeth that met the inclusion criteria were embedded in acrylic resin, the labial surfaces were aligned parallel to the horizontal plane. The middle third of the labial surface was selected for testing as it provides the most flat and uniform surface area, essential for accurate Vickers microhardness measurements [10].

The labial surfaces of these selected specimens were sequentially polished using silicon carbide sandpaper of varying grit sizes (400, 800, 1,000, 1,200, and 2,500) on a rotating polishing machine for 2 seconds, wet polishing

was performed using running water and ultrasonic cleaning for 1 minute between grits until the enamel surfaces become smooth and flat, ideal for accurate microhardness testing. A 2x2 mm^2 window [11] outlined using a scalpel was precisely marked on the labial surface of each tooth to standardize the area designated for subsequent testing procedures (Figure 2).

Figure 2 A 2x2 mm^2 window on the labial surface of the primary tooth

Baseline microhardness measurement

Surface enamel microhardness (SMH) was assessed using a Vickers indenter tester (FM-ARS 9000, Future-Tech Corp., Kanagawa, Japan) under a 100g of force for 15 seconds, Four indentations were made on each specimen with a minimum spacing of 200µm between indentations. The baseline SMH value for each specimen was determined by calculating the mean of the four indentation measurements.Specimens with mean SMH value between 300-350 VHN were selected for the study.[11]

Demineralization process

Each specimen was immersed in a demineralizing solution composed of 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, and 0.05 M acetic acid. The pH of the solution was adjusted to 4.4 using KOH, for four days at 37°C within an incubator shaker (Series 25 Incubator Shaker®, Ramsey, MN) [11,12]. Following the demineralization period, each specimen was thoroughly rinsed with deionized water and gently dried with tissue paper [11, 12].

Surface microhardness measurement post-demineralization

Following demineralization, the SMH of each tooth was reassessed using the same method employed at baseline. The average of four readings provided the post-demineralization SMH values. To ensure consistency in the study's conditions, only specimens exhibiting a mean SMH between 100-150 VHN were selected for further analysis [11,12].

Treatment Application

Specimens were randomly allocated into three groups (SAPF, fluoride varnish, and control) simple random sampling via the lottery method, and treatments were applied following manufacturer-specified clinical procedures

- **SAPF Group:** Specimens were pre-treated with 2% sodium hypochlorite (20 seconds) to remove organic contaminants, followed by 35% phosphoric acid (20 seconds)to create micro-porosities. After rinsing with deionized water and air-drying, Single dose vial of Curodont Repair Plus™ (0.1ml) was applied per specimen and allowed to diffuse for 5 minutes according to manufacturer's instructions.
- **Fluoride Varnish (F) Group:** Specimens were air-dried before application of Duraphat® Varnish. The varnish was pre-weighed (0.005g) for each specimen to ensure standardized application using a micro-brush, following standard clinical protocols without additional surface preparation.
- **Control group:** Specimens received no treatment, serving as a negative control.

Following treatment, all specimens were immersed in artificial saliva, comprising 0.65g KCl, 0.058g $MgCl_2$, 0.165g, $CaCl_2$, 0.804g K_2HPO_4, 0.365g KH_2PO_4, 2g $NaCO_2CH_3$ cellulose, with deionized water added to complete 1 litre [13]. This immersion lasted for 24 hours at 37°C in an incubator shaker.

Subsequently, specimens from the F group, retaining varnish post-immersion, underwent a brushing process and were rinsed with deionized water to remove any remaining varnish.

pH Cycling

All specimens underwent pH cycling to simulate the natural demineralization and remineralization process teeth experience. Each cycle consisted of:

- **Demineralization Phase**: 3 hours of exposure to a demineralizing solution (2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, and 0.05 M acetic acid, with the pH adjusted to 4.7 using 1M KOH) [11,12]. This phase was conducted twice daily.
- **Remineralization Phase**: Between the demineralization phases, specimens were placed for two hours in a remineralizing solution (1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 0.15 M KCl, with pH adjusted to 7.0 using 1M KOH) [11,12].

Following the day's cycles, specimens were left in the remineralizing solution for 16 hours overnight at 37°C, using an incubator shaker. This daily sequence was repeated over seven days to closely mimic the fluctuating conditions of oral environments [9,12].

Post-pH-Cycling Microhardness Measurement:

Post-pH-cycling, specimens were rinsed with deionized water, dried, and measured SMH in the same method as baseline. Four readings per specimen were taken, with their mean calculated to assess treatment effects.

The percentage of surface hardness recovery

The percentage recovery of surface microhardness (% SMHR) was calculated using the mean of microhardness as (% SMHR) = 100 x (microhardness after pH cycling - microhardness after demineralization) / (microhardness at baseline - microhardness after demineralization) [15].

Statistical analysis

Data were processed and analyzed using SPSS version 25. The Shapiro-Wilk test confirmed data normality. Repeated Measures ANOVA was used to evaluate differences in SMH values at baseline, post-demineralization, and post-pH-cycling within each group. One-way ANOVA followed by Bonferroni's post-hoc test was used to compare differences among groups at each stage and for percentage recovery. The significance level was set at 0.05 for all tests

Table 1 Two commercial remineralizing products used in this study

Active ingredients	Trade mark	Manufacturing company
5% Sodium fluoride (2.26% fluoride)	Duraphat® Varnish	Colgate Oral Pharmaceuticals, New York, NY
0.05% Sodium fluoride (0.02% fluoride) with self-assembling peptides (P11-4)	Curodont Repair Plus™	Credentis AG, Windisch, Switzerland

Results

Table 2 displays the means and standard deviations of SMH values at baseline, post-demineralization, and post-pH-cycling for each group. Initial analysis revealed no significant differences in SMH values among the groups at baseline and after demineralization (p>0.05), as illustrated in Figure 3's bar chart.

Post-pH-cycling, the mean SMH values for both the SAPF and F groups showed significant increases. While there were no statistically significant differences between the SMH values of the SAPF and F groups, both were significantly higher than those observed in the control group (p< 0.05).

Table 3 shows the percentage recovery of SMH across the groups. The highest percentage recovery of SMH was observed in the F group, although there were no statistically significant differences when compared to the SAPF group (p=0.818), as depicted in Figure 4's bar chart. In contrast, the percentage recovery of SMH significantly decreased in the control group (p<0.001).

Table 2 SMH at baseline, after demineralization, after pH-cycling

Groups	Surface enamel microhardness in VHN (Mean ± SD)			
	Baseline	Post-demineralization	Post-pH-cycling	P value
SAPF group	334.13±11.63$^{A, a}$	136.80±11.62$^{B, b}$	180.09±7.47$^{C, c}$	p<0.001
F group	331.57±11.72$^{A, a}$	135.59±14.44$^{B, b}$	186.85±10.94$^{C, c}$	p<0.001
Control group (No treatment)	340.98±6.43$^{A, a}$	137.11±9.96$^{B, b}$	117.45±8.17$^{B, b}$	p<0.001
P value	p=0.127	p=0.958	p<0.001	

Repeated ANOVA, One-way analysis of variance (ANOVA) and Bonferroni's method comparison test.
The different capital letters indicate statistically significant differences of the inter-groups in the same column (p < 0.05).
The different small letters indicate statistically significant differences in the intra-group within the same row (p < 0.05).
SD = standard deviation, VHN = Vicker hardness number

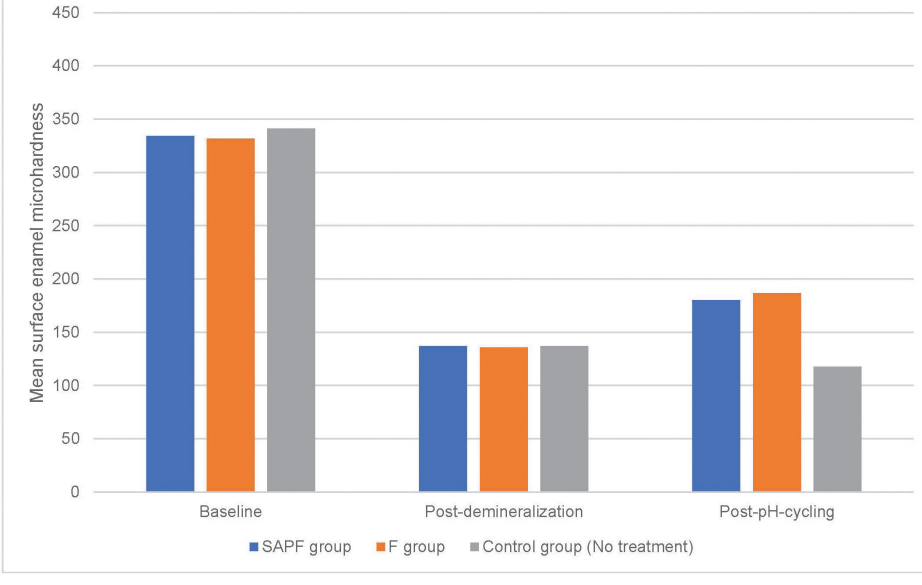

Figure 3 Representative mean surface enamel microhardness in Vicker hardness number at baseline, after demineralization, after pH-cycling

Table 3 The percentage recovery of SMH (%SMHR)

Group	%SMHR (Mean ± SD)
SAPF group	21.93±4.89^{A}
F group	25.75±10.14^{A}
Control group (No treatment)	-9.93±6.86^{B}
p-value	p<0.001

One-way analysis of variance (ANOVA) and Bonferroni's method comparison test.
The different superscript letters indicate statistically significant differences (p< 0.05).

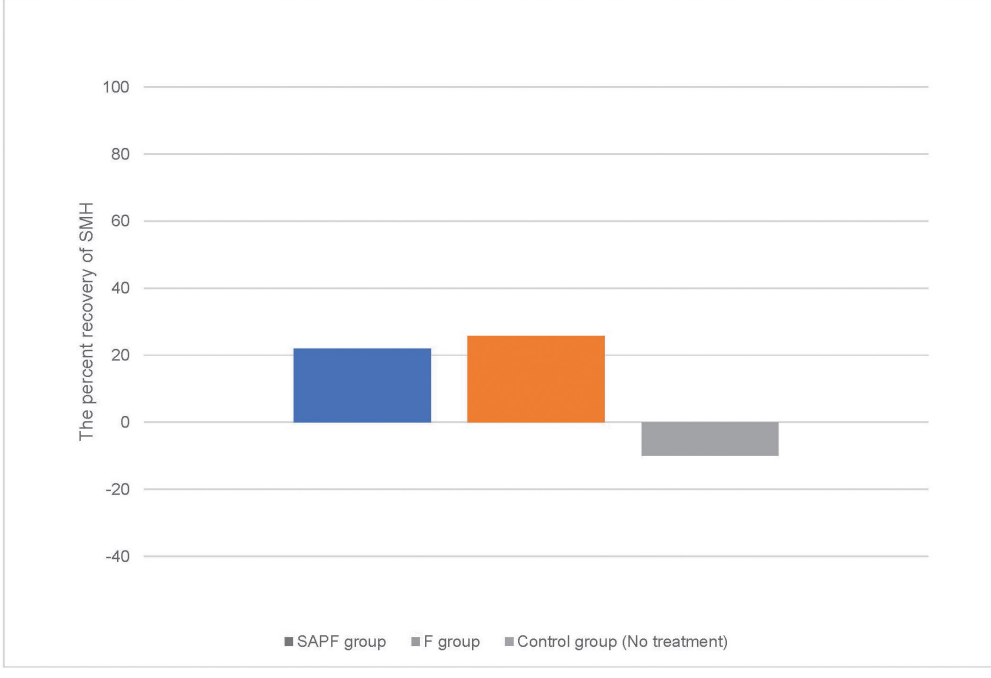

Figure 4 Representative The percentage recovery of SMH (Mean)

Discussion

This study found the baseline microhardness of enamel to be 335.56±10.66 VHN, which was consistent with values reported in prior studies of primary teeth [11,12]. After demineralization, the enamel microhardness decreased to 136.50±11.74, consistent with the findings of Kasemkhun et al., which were 115.56±19.15. No significant differences in microhardness values were observed among groups at baseline and post-demineralization (p>0.05), confirming the uniformity of enamel demineralization across all groups and validating the comparative analysis of material remineralization effects.

Post-pH-cycling, the fluoride group's microhardness was 186.85±10.94, corroborating existing literature. [11,16]. The fluoride group's SMH was significantly higher than that of the control group (p<0.05), consistent with the observations of Rirattanapong et al., which indicated that microhardness values for the 5% NaF varnish group were significantly greater than

those of the control. The positive control in this study confirmed fluoride's well-documented remineralization efficacy. Conversely, the control group's post-pH-cycling microhardness was 117.45±8.17, similar to past findings [11,16], with a -9.93±6.86 percentage recovery of SMH comparable to earlier studies [12,16], highlighting the absence of remineralization in untreated teeth.

This research marks the first investigation into a product combining self-assembling peptides with incorporated fluoride (SAPF), whereas previous studies have only examined sequential applications of these components. Significant increases in SMH were observed in the SAPF group compared to the control ($p < 0.05$), indicating SAPF's remineralizing capability on primary teeth. This finding aligns with Kamal et al.'s conclusion that self-assembling peptides followed by fluoride enhance remineralization and SMH[8], though several methodological differences between the studies merit consideration.

While both studies demonstrated significant remineralization, our study used SAPF containing 0.02% fluoride in a single application, whereas Kamal *et al*. employed sequential applications of self-assembling peptide followed by 2.26% fluoride varnish. We used primary teeth with specific pre-treatment protocols, while Kamal et al. used permanent teeth with different preparation methods. Additionally, our pre-treatment included an etching step before SAPF application to mimic clinical conditions. This etching likely cleared the remaining pseudo-intact surface layer of the lesion, potentially enhancing the penetration of self-assembling peptides. While this etching step is recommended in vivo to remove pellicle and mineral debris, these elements are not present in artificially induced enamel lesions. Furthermore, our study incorporated pH cycling to better simulate oral conditions.

Interestingly, while both studies showed positive results, our findings revealed that SAPF's remineralization efficacy was comparable to fluoride varnish, despite no significant difference in the percentage recovery of SMH after pH-cycling. This differs from Kamal et al.'s findings, where the sequential application of self-assembling peptide and fluoride showed higher remineralization potential than fluoride alone[8]. These variations in outcomes might be attributed to differences in fluoride concentration (0.02% versus 2.26%), suggesting a dose-response relationship [17], as well as differences in application protocols and experimental design, particularly our inclusion of pH-cycling.The underlying mechanism of SAPF helps explain these findings.

The comparable remineralization efficacy of SAPF can be explained through its unique mechanism of action. Self-assembling peptides regenerate enamel within the lesion body by forming a three-dimensional network that simulates the enamel matrix. This process involves the formation of beta-sheet nano tapes, ribbons, fibrils, and fibers. The peptide's negatively charged sites, spaced approximately 9.4 Å apart, serve as potential Ca^{2+} binding sites, matching the columnar Ca^{2+} ions position in the hydroxyapatite (HAP) crystal lattice. This scaffold creates strong chemical bonding with the tooth surface, mimicking enamel matrix proteins' function and providing a template for HAP nucleation and deposition within the lesion.[6] While the peptides create this structural framework, the fluoride component enhances the enamel apatite crystallinity, reducing lesion depth and improving acid solubility resistance. This leads to decreased demineralization rates and increased remineralization [18], ultimately contributing to improved surface microhardness. This synergistic interaction between peptides and fluoride explains how SAPF achieves comparable remineralization

efficacy to fluoride varnish despite its lower fluoride concentration. However, these findings must be interpreted within the context of laboratory conditions

The different surface preparation protocols used for SAPF and fluoride varnish reflect their distinct mechanisms of action. SAPF requires specific surface preparation (sodium hypochlorite and phosphoric acid) to facilitate peptide infiltration into subsurface lesions and enable self-assembly into three-dimensional matrices supporting remineralization [6]. In contrast, fluoride varnish requires no surface preparation as it primarily acts through surface interaction and fluorapatite crystal formation [18]. The post-treatment procedures also differed due to material characteristics: fluoride varnish required mechanical removal after 24 hours due to its visible residual layer, while SAPF, being colorless and fully infiltrative, left no surface residue. While these methodological differences represent standard clinical applications as recommended by their respective manufacturers, they introduce a variable that could influence the remineralization patterns observed.

Considering the controlled lab environment of this study, it's crucial to approach the direct application of these findings to clinical scenarios with caution. The laboratory conditions, though precise for controlled experimentation, don't fully capture the oral cavity's complexity, including the protective influence of saliva and daily habits on dental health [19]. This study, designed with a pH-cycling model, acknowledges several limitations: the absence of bacterial biofilms and natural saliva proteins, and the relatively short duration of pH cycling may not fully represent long-term oral conditions. Furthermore, the exclusive focus on primary teeth, while intentional, raises questions regarding the applicability of these results to adult dentition, which may react differently to the treatments. However, SAPF's lower fluoride concentration (0.02%) offers a potentially advantageous safety profile compared to fluoride varnish, which, while generally safe, has contraindications for specific conditions [20]. Future studies comparing SAPF with other biomimetic remineralization agents and exploring its efficacy across a broader dental spectrum through clinical trials would be valuable to better understand its therapeutic potential.

Conclusion

The study's findings suggest that SAPF could be as effective in remineralizing primary teeth as traditional fluoride varnish. This indicates SAPF's potential as a viable alternative in dental care, particularly in remineralization treatments. Further investigations are necessary to fully understand SAPF's range of applications and benefits in clinical settings.

Conflicting Interest (If present, give more details)

Nil.

Acknowledgement

The authors thank Miss Chayada Teanchai and the senior staff of the Research Service Center of the Faculty of Dentistry, Mahidol University.

Supplementary

Table 4 Supplementary raw data for surface microhardness (SMH) measurements

ID	Group	Surface enamel microhardness in VHN		
		Baseline	Post-demineralization	Post-pH-cycling
4.00	SAPF	341.74	149.68	172.14
5.00	SAPF	319.90	136.20	180.63
7.00	SAPF	331.64	140.43	174.03
8.00	SAPF	348.70	135.21	185.69
12.00	SAPF	320.68	112.70	171.63
15.00	SAPF	346.50	143.18	182.04
19.00	SAPF	327.07	134.43	184.82
21.00	SAPF	335.51	143.34	183.50
24.00	SAPF	321.53	149.91	194.23
28.00	SAPF	348.06	122.96	172.28
2.00	F	331.41	147.66	192.87
3.00	F	319.22	114.44	185.53
6.00	F	323.63	110.91	202.26
10.00	F	345.69	149.82	173.56
11.00	F	324.90	134.93	178.41
16.00	F	339.10	124.76	204.29
17.00	F	320.04	147.35	172.65
18.00	F	348.67	149.50	181.14
23.00	F	343.68	140.38	190.77
25.00	F	319.41	136.19	187.06
1.00	Control group (No treatment)	347.08	139.68	128.55
9.00	Control group (No treatment)	338.78	134.03	113.60
13.00	Control group (No treatment)	341.94	121.34	120.73
14.00	Control group (No treatment)	338.50	147.30	101.99
20.00	Control group (No treatment)	339.94	149.66	116.75
22.00	Control group (No treatment)	344.03	147.75	120.78
26.00	Control group (No treatment)	345.60	127.18	119.68
27.00	Control group (No treatment)	348.20	125.42	106.87
29.00	Control group (No treatment)	340.21	141.40	126.53
30.00	Control group (No treatment)	325.53	137.32	119.06

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