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Remineralization effects of self-assembling peptide P₁₁-4 associated with three materials on early enamel carious lesions: An in vitro study

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Abstract

The aim of this study was to assess the remineralization of enamel caries lesions using the self-assembling peptide P₁₁-4 associated with different materials. Artificial early enamel lesions were prepared on 154 primary teeth. The samples were randomly divided into eight groups: (1) control, (2) P₁₁-4, (3) fluoride toothpaste (FT), (4) P₁₁-4 + FT, (5) casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), (6) P₁₁-4 + CPP-ACP, (7) fluoride bioactive glass toothpaste (BT), and (8) P₁₁-4 + BT. The surface enamel microhardness (EMH) and energy-dispersive X-ray spectroscopy (EDS) of the teeth were then measured at the baseline, after demineralization, and after 28 days of remineralization. The enamel surfaces were assessed by field emission scanning electron microscopy (FESEM) and atomic force microscopy (AFM). The data were analyzed with one-way analysis of variance (ANOVA) ($p < .05$). EMH after demineralization was significantly lower than the baseline value ($p < .001$). The interventions led to an enhanced percentage of EMH recovery (%REMH), which was higher in Groups 6 and 7. There was no significant difference between Groups 3 and 4. Groups 1 and 2 had the lowest %REMH. The mean calcium/phosphate weight percentage ratio of P₁₁-4 was significantly lower than the others ($p < .001$). The FESEM and AFM images revealed mineral deposition on the eroded enamel and reductions in surface roughness in Groups 5 and 7.

KEYWORDS

bioactive glass, CPP-ACP, remineralization, roughness, self-assembling peptide

1 | INTRODUCTION

Caries begin with inorganic loss (demineralization) in the tooth, and a cavity will develop if the minerals are not replaced (remineralization) (Featherstone, 2008). Treatments of noncavitated early caries lesions are divided into two main categories: micro-invasive strategies or using non-invasive materials to remineralize the enamel (Philip, 2019; Schwendicke et al., 2019). Traditionally, fluoride products form fluorapatite and effectively reverse caries by improving enamel remineralization (Philip, 2019).

Hydroxyapatite crystals are composed mainly of calcium (Ca) and phosphate (PO₄³⁻) ions; thus, materials that contain these ions, such

as casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), have been developed. The material “stabilizes amorphous calcium phosphate (ACP) and forms CPP-ACP complexes,” leading to enhanced Ca and PO₄³⁻ in dental plaque and increased remineralization (Dai et al., 2019; Oliveira, Barboza, Barreto, & Tostes, 2020; Shen et al., 2020). There is still controversy about the superiority of CPP-ACP compared to fluoride products to treat caries lesions. A greater efficacy of CPP-ACP than fluoride was suggested by reports of in vitro studies that did not follow pH-cycling conditions in permanent (Shen et al., 2020) or primary teeth (Zhang, Zou, Yang, & Zhou, 2011), as well as in a clinical study in permanent teeth

(Dai et al., 2019). However, the others did not report the effect (Oliveira et al., 2020; Vyavhare, Sharma, & Kulkarni, 2015).

Bioactive glass is a biocompatible material that has proven advantages in preventive dentistry. BioMin F is a bioactive glass toothpaste that contains 5% fluoro calcium phosphosilicate. The ions release slowly, based on “smart technology” and form apatite, which enhances enamel remineralization (Brauer, Karpukhina, O'Donnell, Law, & Hill, 2010; Taha, Patel, Hill, & Fleming, 2017). Some studies recommended BioMin F toothpaste to treat early caries lesions compared to fluoride toothpaste in permanent teeth (Alhussain, Alhaddad, Ghazwi, & Farooq, 2018; Bakry et al., 2018).

Remineralization of early caries lesions is another alternative based on tissue regeneration because it mimics the physiological function of the enamel matrix. Self-assembling peptide P₁₁₋₄ forms a biomatrix scaffold in the body of the lesion and provides a suitable area to form new hydroxyapatite (Hartgerink, Beniash, & Stupp, 2001; Kind et al., 2017; Kirkham et al., 2007; Philip, 2019). Some studies revealed greater efficacy of P₁₁₋₄ in the remineralization of early caries lesions in permanent teeth than CPP-ACP or fluoride varnish that were assessed in other in vivo (Brunton et al., 2013) and in vitro studies (Sindhura, Uloopi, Vinay, & Chandrasekhar, 2018). Other clinical studies (Alkilzy, Tarabaih, Santamaria, & Splieth, 2018; Jablonski-Momeni, Nothelfer, Morawietz, Kiesow, & Korbmacher-Steiner, 2020) and laboratory research on permanent teeth concluded that remineralization is enhanced after applying P₁₁₋₄ in conjunction with CPP-ACP or fluoride varnish (Kamal, Hassanein, Elkassas, & Hamza, 2020).

It is important to use noninvasive methods to treat early caries lesions in young children. With respect to P₁₁₋₄ mechanism, our study aimed to assess providing enamel regeneration followed by use of other remineralizing agents. Our hypothesis was that P₁₁₋₄ in combination with CPP-ACP or fluoride agents to remineralize enamel would be similar or superior to applying these materials alone, because of the synergistic effect of each. The null hypothesis was tested against an alternative hypothesis that differences in remineralizing would be found between the materials. To our knowledge, there are no studies that assess the influence of P₁₁₋₄ and compare these noninvasive preventive methods in primary teeth. Therefore, the aim of the current study was to evaluate fluoride-based toothpaste, fluoride-containing bioactive glass toothpaste, and CPP-ACP with or without self-assembling peptide P₁₁₋₄ for the remineralization of artificial enamel caries lesions in primary teeth. Surface enamel microhardness (EMH), energy-dispersive X-ray spectroscopy (EDS), field emission scanning electron microscopy (FESEM), and atomic force microscopy (AFM) assessments of the lesions were compared after treatment with these materials.

2 | MATERIALS AND METHODS

The Human Ethics Review Committee of the School of Dentistry, Shiraz University of Medical Sciences approved this in vitro study. We confirm that all methods were carried out in accordance with relevant guidelines and regulations. In total, 160 human primary canine teeth were extracted due to orthodontic treatment from 6 to 8 years old children. The teeth were kept in distilled water. The parents signed an

informed consent form that authorized the use of these teeth for research purposes.

2.1 | Sample preparation

The specimens were disinfected by soaking in 0.1% Chloramine T solution for 30 days. Then all the crowns were assessed under a stereomicroscope to discard teeth with any cracks, anomalies, stains, or defects. From these, we chose 154 teeth and their roots were removed to the level of 1 mm under the enamel-dentin junction. The enamel surfaces were prepared by using silicon carbide papers (range: 600–2,000 grit), followed by the application of aluminum oxide (0.5–3 µm), and finally rinsed with distilled water. Then, the teeth were randomly allocated to eight groups as follow; control ($n = 14$) and interventinal groups ($n = 20$). Demineralization and remineralization solution were also prepared as follows:

- Early caries lesions process: Each sample was immersed in 30 mL demineralization solution, at 37°C for 96 hr. The solution contained 0.1 mM lactic acid solution, 3 mM CaCl₂, 3 mM KH₂PO₄, 0.2% guar gum (pH 4.5). The final pH was adjusted to 4.5 with 50% sodium hydroxide (NaOH). The solutions were replaced with new ones after 48 hr. After 96 hr, each specimen was washed with deionized water for 20 s, air-dried and a second EMH test performed (Patil, Choudhari, Kulkarni, & Joshi, 2013; Zhang et al., 2011).
- Remineralizing solution: The artificial saliva solution consisted of 2.200 g/L gastric mucin, 0.381 g/L sodium chloride (NaCl), 0.213 g/L calcium chloride (CaCl₂·2H₂O), 0.738 g/L potassium hydrogen phosphate (K₂HPO₄·3H₂O), and 1.114 g/L potassium chloride (KCl). The final pH was adjusted to 7.00 at 37°C with 85% lactic acid (Patil et al., 2013). We replaced the freshly prepared artificial saliva daily in order to overcome the risk of mucin structural changes during the intervention phase. The pH level was monitored in the beginning and at the end of each daily intervention. The samples that contained the artificial saliva were incubated at 37°C in order to better simulate oral cavity conditions.

2.2 | Experimental protocols

The following treatment was performed on the demineralized enamel in each group:

- Group 1 (control): Samples with polished enamel surfaces were immersed in artificial saliva solution and received no interventions. The solution was replaced every day for 28 days.
- Group 2 (self-assembling peptide P₁₁₋₄): The procedure was performed according to the manufacturer's instructions. After tooth demineralization, the enamel surfaces were cleaned with a brush, rinsed with 2% sodium hypochlorite (Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany) for 20 s, and washed with distilled water for 30 s. Then, the enamels were etched with 37% phosphoric acid (3 M, ESPE, St. Paul, MN) for 20 s, rinsed with distilled

water, and allowed to air dry. The peptide P₁₁-4 is available as Curodont powder (Curodont Repair, Credenits AG, Windisch, Switzerland). A glass vial of Curodont powder was dissolved in 0.05 mL of deionized water and immediately applied to the surfaces of the teeth for 5 min to allow for penetration into the eroded enamels. The samples were subsequently placed in a remineralization solution (artificial saliva), which was changed every 24 hr. Only one application of Curodont was used for the entire study.

- Group 3 (fluoridate toothpaste): Totally 0.25 g of 1,450 ppm fluoride toothpaste (Oral B, Iowa) applied as a thin layer to the surface of each tooth. Each sample was immersed in 30 mL of artificial saliva and stirred at 100 rpm for 3 min. Then, the tooth was rinsed with deionized water for 20 s and immersed in a remineralization solution. This procedure was performed twice daily at 8 a.m. and 3 p.m., after which the enamel was rinsed with deionized water for 20 s.
- Group 4 (P₁₁-4 + fluoridate toothpaste): Eroded enamel was first treated with P₁₁-4 then 1,450 ppm fluoridate toothpaste, as described for Groups 2 and 3. During 28 days of the study, the procedure was performed twice per day, as mentioned for Group 3.
- Group 5 (CPP-ACP): Enamel surface was covered with a thin layer of water-based, sugar-free topical cream (tooth mousse) that contained Recaldent (GC, Tokyo, Japan), and subsequently stirred at 100 rpm for 3 min in artificial saliva. The remainder of the process was performed as described for Group 3, twice per day for 28 days.
- Group 6 (P₁₁-4 + CPP-ACP): P₁₁-4 was initially applied to the enamel, followed by CPP-ACP as described for Groups 2 and 5. The previously described procedure for Group 5 was repeated twice per day for this group along 28 days of the study.
- Group 7 (fluoridate bioactive glass toothpaste): Totally 0.25 g of BioMin F toothpaste (BioMin Technologies, Ltd., London, UK) that contained Ca and fluoride (<600 ppm) was applied to the eroded enamel and the teeth were subsequently immersed in 30 mL of artificial saliva and stirred at 100 rpm for 3 min. The remainder of the process was performed as described for Group 3, twice a day for 28 days.
- Group 8 (P₁₁-4 + fluoridate bioactive glass toothpaste): The specimens were treated with P₁₁-4 and BioMin F according to the procedures for Groups 2 and 7. The remainder of the process was performed as described for Group 3, two times a day for 28 days.

The materials and methods used in the study shown in Table 1 and Figure 1.

All the samples were subjected to laboratory tests at baseline, after demineralization, and 28 days after enamel treatment followed by rinsing with deionized water for 20 s.

2.3 | Microhardness test

A total of 80 teeth were embedded in acrylic blocks with placement of the buccal surface of each tooth at the top surface ($n = 10$). We used

TABLE 1 Materials used in the current study

Material	Chemical composition	Manufacturer
Curodont repair (P ₁₁ -4)	Monomeric self-assembling peptides (P ₁₁ -4)	Credenits AG, Windisch, Switzerland
Fluoridate toothpaste	Sorbitol, aqua, hydrated silica, cocamidopropyle betaine, trisodium phosphate, aroma, cellulose gum, sodium phosphate, sodium fluoride, carbomer, sodium saccharine, 1,450 ppm fluoride	Oral B, Iowa, USA
CPP-ACP (tooth mousse, Recaldent)	Pure water, glycerol, CPP-ACP, D-sorbitol, CMC-Na, propylene glycol, silicone dioxide, titanium dioxide, xylitol, phosphoric acid, zinc oxide, sodium saccharin, ethyl p-hydroxybenzoate, magnesium oxide, guar gum, propyl p-hydroxybenzoate, butyl p-hydroxybenzoate	GC, Tokyo, Japan
BioMin F (fluoride containing bioactive glass toothpaste)	Glycerin, silica, PEG 400, fluoro calcium phosphosilicate, sodium lauryl sulphate, titanium dioxide, aroma, carbomer, potassium acesulfame, fluoride content 530 ppm	BioMin Technologies, Ltd., London, UK

paper adhesive to generate a 2 × 2 mm window on the surface of each tooth and covered the surrounding area with two layers of nail polish in order to create an equal test area. A Vickers diamond indenter (SCTMC DHV-1000Z, digital microhardness tester, Shanghai, China) was used to determine the EMH at a 50 g force for 15 s, at five points, and a distance of 100 µm. The average of these five points was recorded. EMH was measured at baseline, after demineralization, and remineralization. The baseline EMHs of the specimens were 315.03 to 324.83 Vickers hardness number (VHN). After demineralization, the surfaces were rinsed with distilled water for 20 s and dried. We then calculated the EMH for this time point. After obtaining the last EMH, the percent EMH recovery (%REMH) in the remineralized enamel was calculated as follows: %REMH = (remineralized EMH – demineralized EMH)/(sound tooth microhardness – demineralized EMH) × 100.

2.4 | Energy-dispersive X-ray spectroscopy and field emission scanning electron microscopy

We selected 42 teeth from the interventional groups ($n = 6$) to measure the minerals at the surface layer of each tooth at three time points: baseline, after demineralization, and remineralization. The procedure

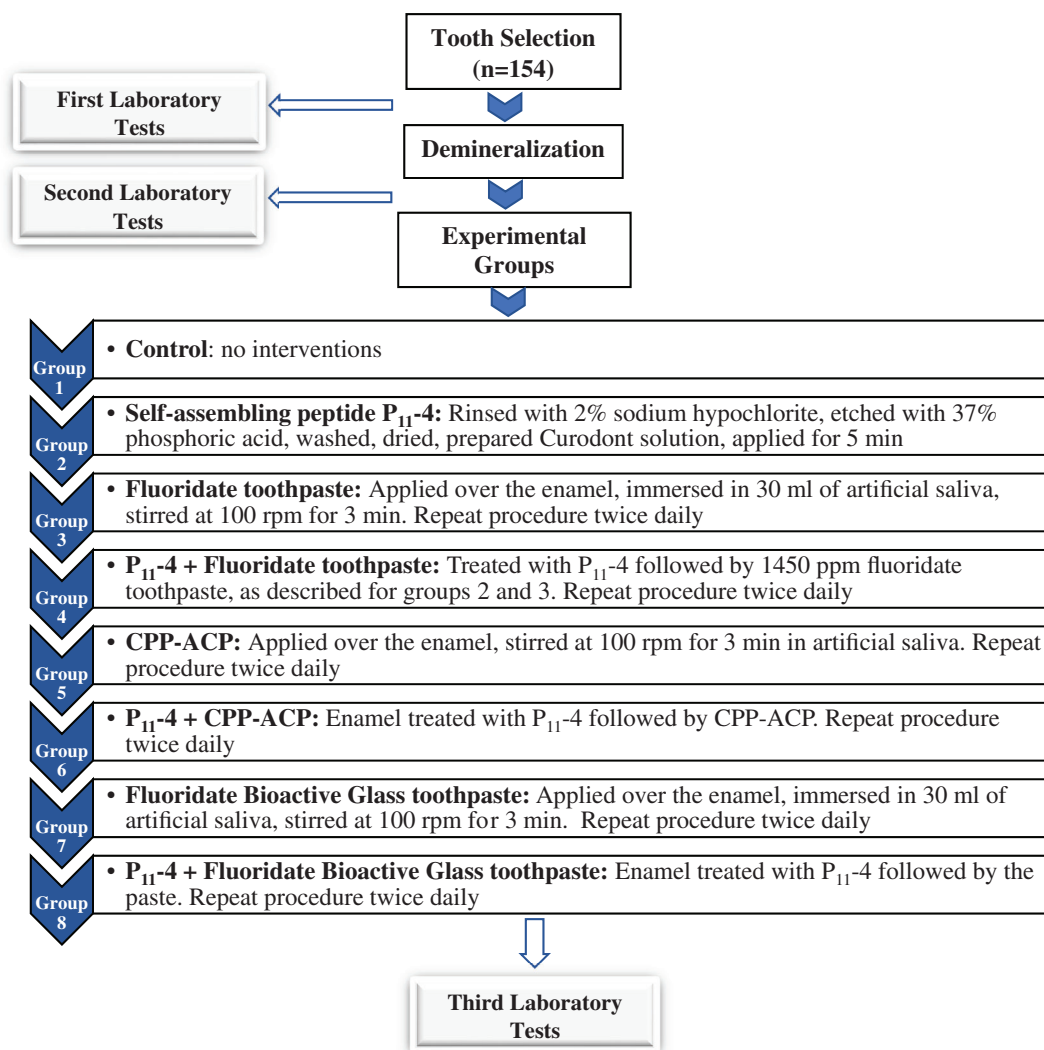


FIGURE 1 Flowchart showing the methods and materials used in this study

was conducted according to former studies and analytic method. Energy-dispersive X-ray spectroscopy (EDS; spot size: 100 nm) in conjunction with high-resolution field emission scanning electron microscopy (FESEM; spot size: 2–3 nm; MIRA3, Tescan, Brno, Czech Republic) was used to assess the surfaces of the teeth. The enamel surfaces were gold-coated and the surfaces were scanned by FESEM at 15 kV for 45 s, and magnifications of 35,000 \times and 75,000 \times .

2.5 | Atomic force microscopy

The surfaces of 32 teeth ($n = 4$) were evaluated with an Atomic force microscopy (AFM) device (JPK Nanowizard II apparatus, JPK Instruments, Berlin, Germany) in identified areas as described above: after enamel polishing, demineralization, and treatment with the study materials. The analysis was performed by scanning in the tapping mode with nonconductive silicon nitride at a speed of 1 Hz. The surface root mean square roughness (Ra) was calculated for each sample in a $5 \times 5 \mu\text{m}^2$ area for five areas.

2.6 | Statistical analysis

IBM SPSS for windows version 22.0 (IBM Corp., Armonk, NY) was used for data analysis. All data are presented as mean \pm standard deviation (SD). For between-group comparisons, one-way ANOVA and Tukey's HD test were separately used for each condition and the mean %REMH between the groups. One-way ANOVA and Tukey's post hoc tests were used to compare the effect of different materials and conditions in terms of mineral content. p values $< .05$ were considered to be statistically significant.

3 | RESULTS

3.1 | Enamel microhardness

There was significant interaction between the conditions and the materials ($p = .024$). Table 2 shows the mean \pm SD for microhardness and %REMH in the polished, demineralized, and remineralized enamel.

TABLE 2 Comparison of enamel surface microhardness in the experimental groups

Group (n = 10)	Condition (mean \pm SD)			%REMH
	Baseline	DEM	REM	
1. Control	319.08 \pm 2.01 ^{A,a}	131.52 \pm 2.40 ^{A,b}	133.84 \pm 2.23 ^b	0.85 \pm 1.60 ^A
2. Curodont	319.68 \pm 2.02 ^{A,a}	132.82 \pm 2.90 ^{A,b}	176.19 \pm 20 ^c	23.19 \pm 1.58 ^B
3. Toothpaste	318.88 \pm 2.81 ^{A,a}	134.42 \pm 2.45 ^{A,b}	246.01 \pm 4.90 ^c	60.50 \pm 3.44 ^C
4. Curodont + toothpaste	319.38 \pm 2.69 ^{A,a}	133.72 \pm 2.34 ^{A,b}	248.6 \pm 4.88 ^c	61.88 \pm 2.37 ^C
5. CPP-ACP	318.68 \pm 2.58 ^{A,a}	132.84 \pm 2.23 ^{A,b}	259.38 \pm 5.98 ^c	68.06 \pm 3.20 ^D
6. Curodont + CPP-ACP	321.26 \pm 2.56 ^{A,a}	133.46 \pm 2.33 ^{A,b}	294.12 \pm 7.16 ^c	85.57 \pm 3.90 ^E
7. BioMin F	320.76 \pm 2.90 ^{A,a}	133.45 \pm 2.95 ^{A,b}	279.47 \pm 2.27 ^c	77.97 \pm 1.91 ^F
8. Curodont + BioMin F	320.86 \pm 2.57 ^{A,a}	134.22 \pm 2.25 ^{A,b}	218.07 \pm 4.06 ^c	44.94 \pm 2.44 ^G
p value	.112	.286	<.001	<.001

Note: Mean values with the same lowercase letter in each row are not statistically significant. Mean values with the same capital letter in each row are not significantly different.

Abbreviations: CPP-ACP, casein phosphopeptide-amorphous calcium phosphate; DEM, demineralization; %REMH, percentage microhardness recovery in remineralized teeth; REM, remineralization.

The mean EMH at the beginning of the study was 319.9 \pm 2.52 VHN. There was no significant difference observed between the groups at baseline ($p = .112$). After demineralization, we noticed a significant reduction in the EMH of the groups (all $p < .001$). However, there was no significant difference observed among the groups in the demineralization phase ($p = .286$). Application of the remineralizing materials significantly enhanced EMH (all $p < .001$). The P₁₁-4 group had the least mean %REMH followed by the P₁₁-4 + bioactive glass toothpaste group. There was a significantly greater increase in %REMH in the P₁₁-4 + CPP-ACP group, followed by the bioactive glass toothpaste and CPP-ACP groups compared with the other groups (all $p < .001$). However, there was no significant difference in enhancing %REMH between the P₁₁-4 + toothpaste and the toothpaste groups ($p = .940$). The smallest change in %REMH was in the control group, which was significantly different from the other groups (all $p < .001$).

3.2 | Mineral content

We measured the values of Ca and PO₄³⁻ in the treated teeth at baseline, demineralization, and remineralization by EDS. Table 3 shows the mean \pm SD Ca/PO₄³⁻ weight percentage (wt%) ratio for each material. All groups showed significant differences between the conditions (all $p < .05$). The highest ratio was measured at baseline and the lowest was in the eroded samples. There was a statistically significant difference between these materials in remineralization in terms of the Ca/PO₄³⁻ wt% ratio ($p < .001$). The mean Ca/PO₄³⁻ wt% ratio for P₁₁-4 was significantly less than the other experimental groups ($p < .001$). However, the other groups did not show significant differences in the mean Ca/PO₄³⁻ wt% ratio ($p > .05$).

3.3 | FESEM analysis

The eroded enamel had high surface porosity and surface roughness and the prism core had dissolved in some areas. Interprism that had a

hydroxyapatite arrangement pattern was observed (Figure 2a,b). Treatment with P₁₁-4 resulted in the formation of crystal-like structures; however, the orientation of the crystals had an irregular pattern (Figure 2c,d). Toothpaste alone or with P₁₁-4 resulted in agglomeration of minerals that were superficially deposited and had an irregular surface (Figure 2e,f). The application of CPP-ACP filled the porous areas and voids, and there was more precipitation of the minerals over the eroded enamel compared with the toothpaste groups (Figure 2g, h). Enamel treated with toothpaste or CPP-ACP associated with P₁₁-4 had more mineral deposition, which was dispersed over the eroded enamel (Figure 2i-l). Images of the bioactive glass toothpaste groups showed precipitation of minerals and decreased enamel porosity; however, in some areas the prism cores were not filled by the paste (Figure 2m-p).

3.4 | AFM observations

The AFM findings demonstrated a mean Ra in the demineralized enamel of 267 \pm 3.42 nm. Surface treatment with various remineralizing materials decreased roughness of the enamel surface to some extent. The demineralized enamel treated with P₁₁-4 had the roughest enamel surface (254.67 \pm 4.13 nm), followed by toothpaste (164.86 \pm 2.24 nm), P₁₁-4 + toothpaste (135.83 \pm 3.43 nm), P₁₁-4 + CPP-ACP (106.9 \pm 4.71 nm), and P₁₁-4 + bioactive glass toothpaste (81.36 \pm 2.02 nm) in the treated teeth. The CPP-ACP and bioactive glass toothpaste groups had the smallest Ra values (75.18 \pm 3.66 nm and 76.42 \pm 2.51 nm, respectively). The treated enamel topography in the P₁₁-4 intervention groups is shown in Figure 3.

4 | DISCUSSION

The current results agree with previous studies that showed the effectiveness of remineralizing agents on surface changes and EMH

TABLE 3 Comparison of Ca/PO₄³⁻ wt% ratio in the experimental groups

Groups (n = 6)	Condition (mean ± SD)		
	Baseline	DEM	REM
2. Curodont	2.40 ± 0.06 ^{A,a}	1.70 ± 0.05 ^{A,b}	1.82 ± 0.09 ^{A,c}
3. Toothpaste	2.40 ± 0.08 ^{A,a}	1.76 ± 0.08 ^{A,b}	2.10 ± 0.01 ^{B,c}
4. Curodont + toothpaste	2.37 ± 0.03 ^{A,a}	1.79 ± 0.06 ^{A,b}	2.13 ± 0.03 ^{B,c}
5. CPP-ACP	2.40 ± 0.02 ^{A,a}	1.81 ± 0.05 ^{A,b}	2.09 ± 0.05 ^{B,c}
6. Curodont + CPP-ACP	2.40 ± 0.03 ^{A,a}	1.74 ± 0.14 ^{A,b}	2.11 ± 0.03 ^{B,c}
7. BioMin F	2.42 ± 0.03 ^{A,a}	1.70 ± 0.07 ^{A,b}	2.10 ± 0.04 ^{B,c}
8. Curodont + BioMin F	2.40 ± 0.03 ^{A,a}	1.75 ± 0.06 ^{A,b}	2.11 ± 0.06 ^{B,c}
p value	.810	.101	<.001

Note: Mean values with the same lowercase letter in each row are not statistically significant. Mean values with the same capital letter in each column are not significantly different.

Abbreviations: Ca/PO₄– wt%, calcium to phosphate weight percentage; CPP-ACP, casein phosphopeptide–amorphous calcium phosphate; DEM, demineralization; REM, remineralization.

(Alhussain et al., 2018; Dai et al., 2019; Schmidlin, Zobrist, Attin, & Wegehaupt, 2016; Zhang et al., 2011).

Microhardness is an easy, nondestructive technique that has been used to assess the effectiveness of remineralizing materials on early caries lesions (Dai et al., 2019; Kamal et al., 2020; Schmidlin et al., 2016). We used specimens that had a baseline EMH range that was similar to previous studies (Schmidlin et al., 2016; Zhang et al., 2011). After demineralization, we noted a significant reduction in EMH values compared to baseline, due to mineral loss from the enamel. However, the %REMH increased after the interventions. The control group did not undergo any treatment and had lowest EMH value, which agreed with previous studies (Alhussain et al., 2018; Kamal et al., 2020; Schmidlin et al., 2016).

Like others, we found that fluoride toothpaste enhances EMH (Dai et al., 2019; Oliveira et al., 2020; Zhang et al., 2011). This might be related to the formation of fluorapatite on the enamel surface. However, the %REMH was lower than in the materials that contained Ca and PO₄³⁻ ions. Two factors might influence the results: a low amount of fluoride that diffused into the subsurface area (Mehta, Nandlal, & Prashanth, 2013), and the need for additional Ca and PO₄³⁻ ions per one unit of fluorapatite (Ca₁₀(PO₄)₆F₂) (Philip, 2019).

Our results showed a higher %REMH after the application of CPP-ACP compared to fluoride toothpaste which agreed with previous studies (Dai et al., 2019; Shen et al., 2020; Zhang et al., 2011). This might be related to the composition of the CPP-ACP, which functioned as a reservoir of Ca and PO₄³⁻ ions, and its ability to penetrate the subsurface lesions to form hydroxyapatite (Mehta et al., 2013). However, some researchers believe that there is a greater influence of fluoride than CPP-ACP on early caries lesions (Oliveira et al., 2020; Vyavhare et al., 2015).

In this study, there was a higher %REMH after treatment with fluoride bioactive glass toothpaste compared with the control, CPP-ACP, and fluoride toothpaste groups, which was in accordance with other studies (Alhussain et al., 2018; Bakry et al., 2018). Bioactive dissolves after contact with saliva and releases Ca, PO₄³⁻, and silica ions

in the outer layer (Krishnan & Lakshmi, 2013). This outer layer provides a source of minerals for remineralization of subsurface lesions by forming hydroxycarbonated apatite (Bakry et al., 2018; Krishnan & Lakshmi, 2013). Since BioMin F has fluoride incorporated inside its composition, it also forms fluorapatite.

The efficacy of a self-assembling peptide in enamel remineralization has been reported (Kamal et al., 2020; Kirkham et al., 2007; Sindhura et al., 2018). The monomeric form with low viscosity diffuses into the demineralized enamel to form a network that leads to new hydroxyapatite nucleation sites (Hartgerink et al., 2001; Kind et al., 2017; Kirkham et al., 2007). Two studies reported increased enamel remineralization by P₁₁-4 than CPP-ACP and fluoride varnish (Sindhura et al., 2018; Üstün & Aktören, 2019). Our results showed that P₁₁-4 led to an increase in EMH compared to the control group; however, %REMH was the lowest among the intervention groups. This may be related to its monomeric composition without Ca or PO₄³⁻ ions. Also, we used only one application of P₁₁-4, which may have affected the results (Wierichs, Kogel, Lausch, Esteves-Oliveira, & Meyer-Lueckel, 2017).

As in the present study, Kamal et al. (2020) found that P₁₁-4 combined with CPP-ACP led to increased EMH. The monomer network comprises amino acids which increase the affinity for Ca and PO₄³⁻ ions and create binding sites for these ions (Brunton et al., 2013; Hartgerink et al., 2001; Kind et al., 2017; Kirkham et al., 2007). These effects may make more rapid, enhanced hydroxyapatite crystallization possible compared to CPP-ACP alone (Kamal et al., 2020). The results of one study showed the effectiveness of the combination of the P₁₁-4 and hydroxyapatite in whitening discolored enamel (Hojabri, Kaisarly, & Kunzelmann, 2020). In clinical studies, an increased efficacy of the fluoride varnish associated with P₁₁-4 compared to fluoride alone was reported (Alkilzy et al., 2018; Jablonski-Momeni et al., 2020). However, the present study did not show any significant advantage with the use of P₁₁-4 prior to fluoride or BioMin F toothpaste, which might be related to the composition of the materials. Fluoride ion and silicate (Si(O)₄)⁴⁻ do not bind at these sites for Ca ions because both are highly negatively charged. Other factors that

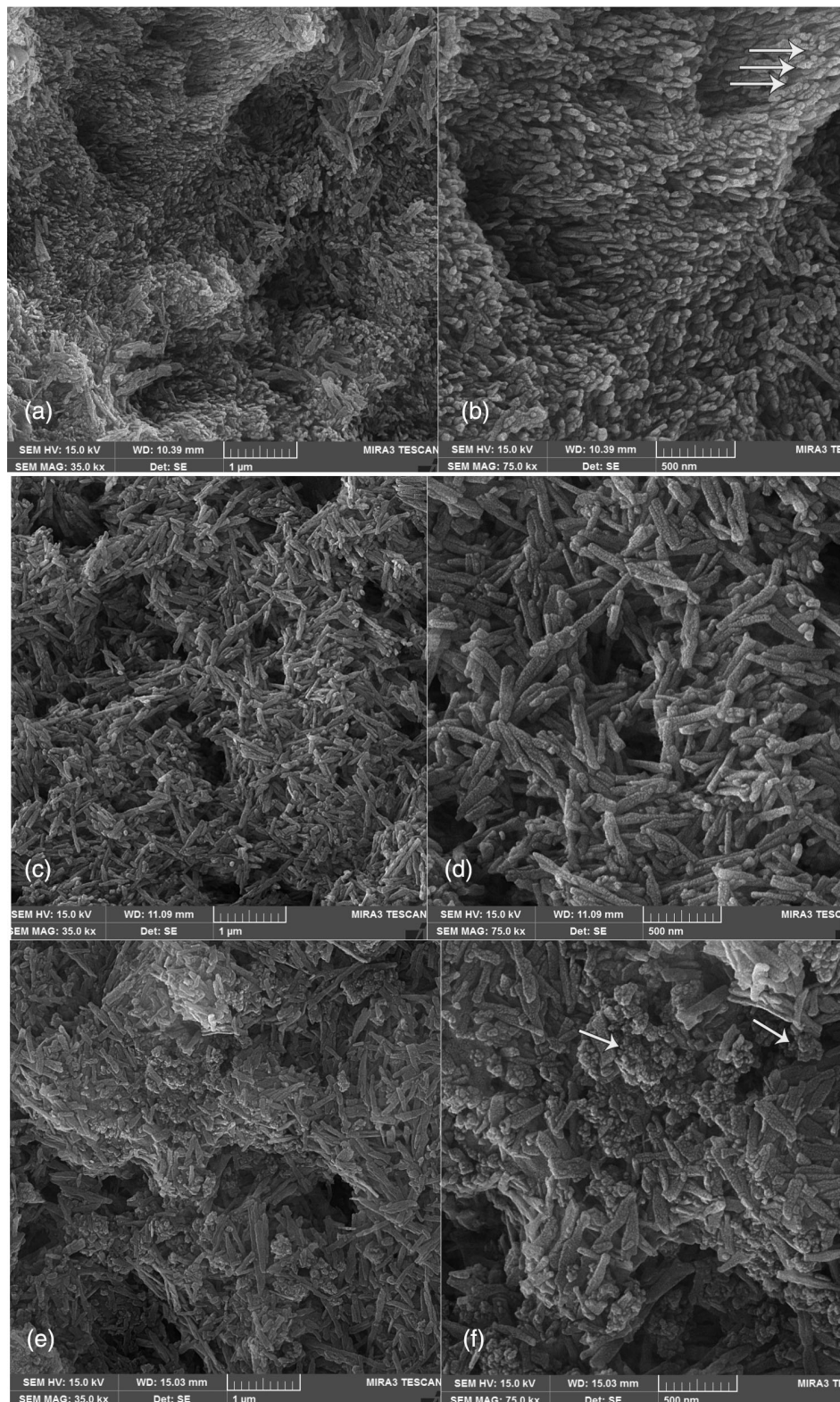


FIGURE 2 Field emission scanning electron microscopy (FESEM) images of the enamel surfaces after: (a, b) demineralization shows an arrangement pattern of hydroxyapatite (arrow), (c, d) application of Curodont, (e, f) application of toothpaste showing mineral agglomeration (arrow), (g, h) application of CPP-ACP showing mineral agglomeration (arrow), (i, j) application of Curodont + toothpaste showing mineral agglomeration (arrow), (k, l) Curodont + CPP-ACP showing mineral dispersal across the demineralized enamel (arrow), (m, n) application of BioMin F and (o, p) Curodont + BioMin F showing mineral deposition on the tooth surface (white arrow). Black arrows show untreated. Magnification: 35,000 \times and 75,000 \times

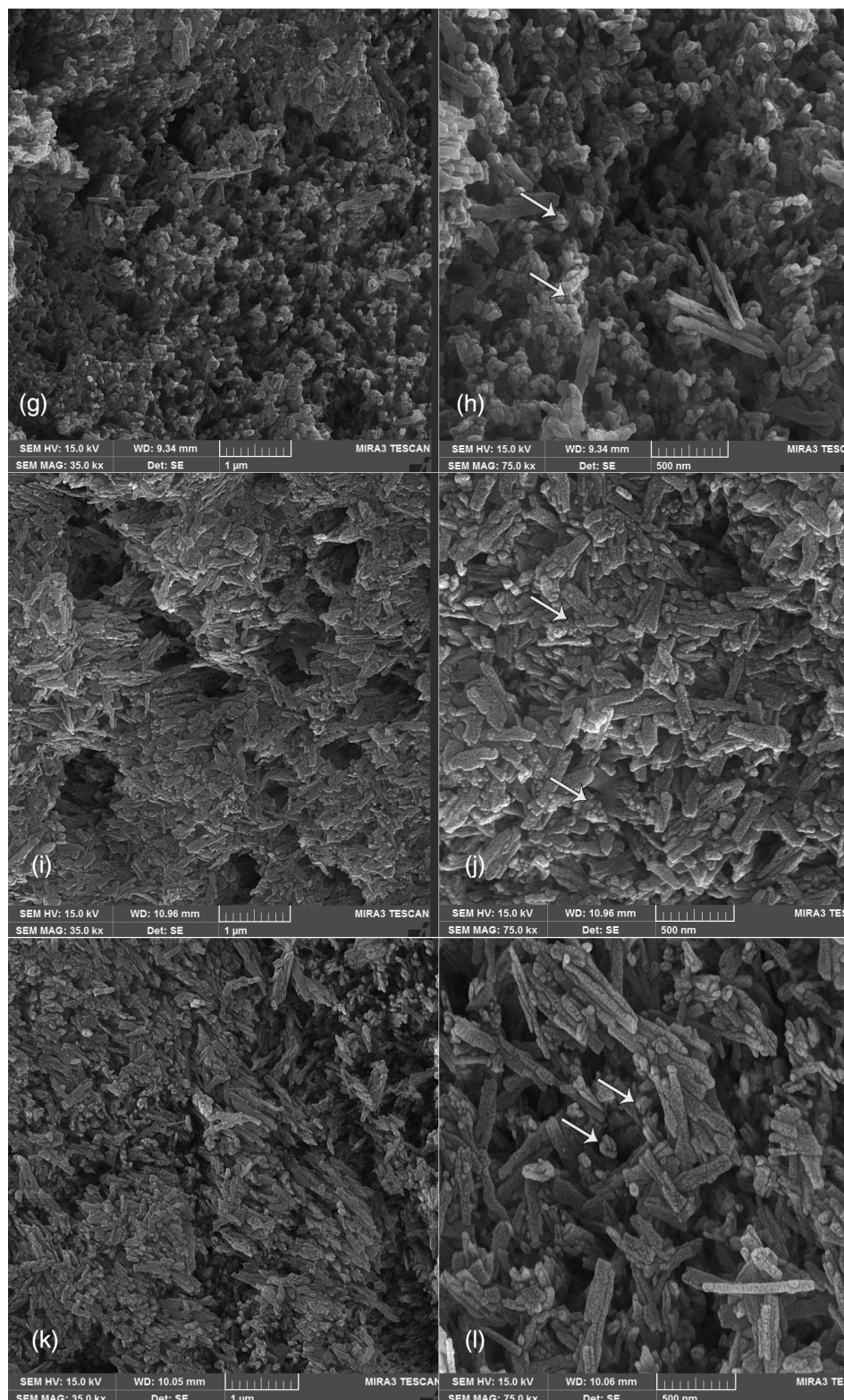


FIGURE 2 (Continued)

might influence the different study results pertain to methodologies and include application times, study duration, and lesion characteristics (Kamal et al., 2020; Kind et al., 2017; Sindhura et al., 2018).

In this research we used the EDS method, as in previous studies, to identify the amount of elements in the early caries lesion after intervention (Bakry et al., 2018; Sindhura et al., 2018). Our results

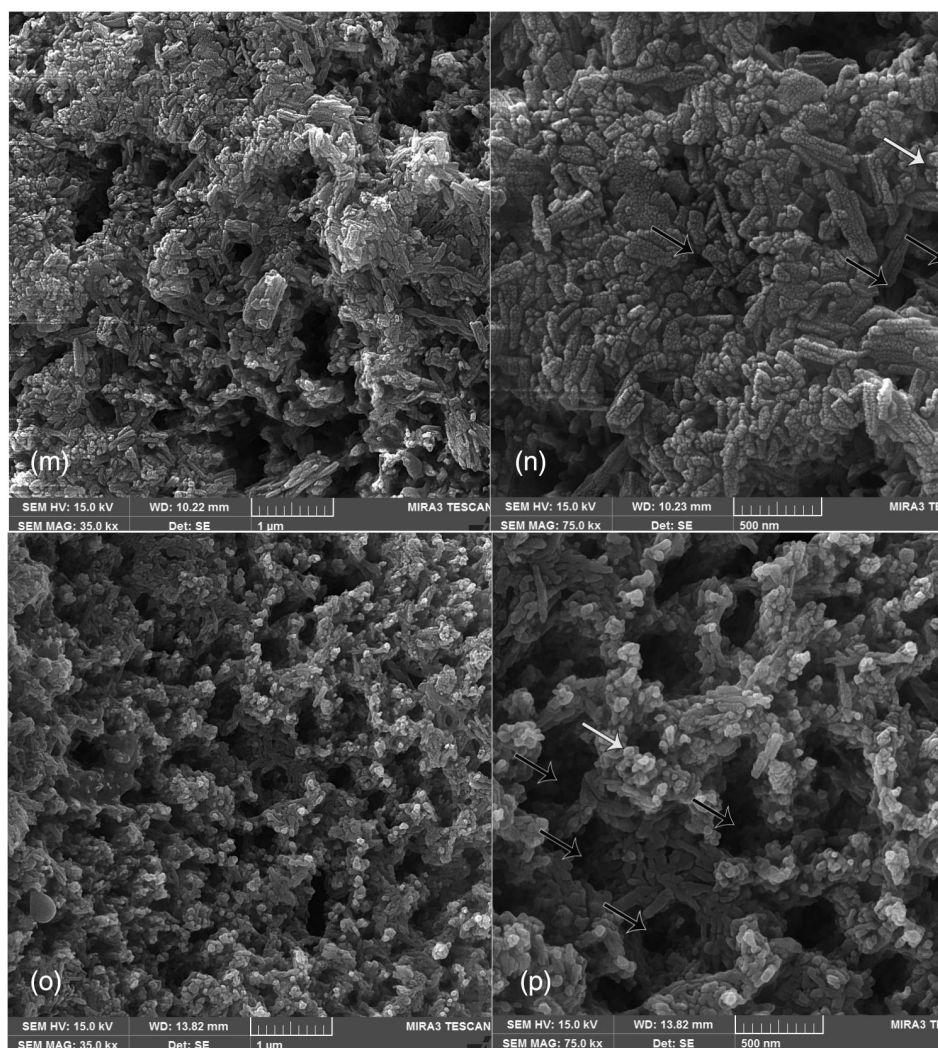


FIGURE 2 (Continued)

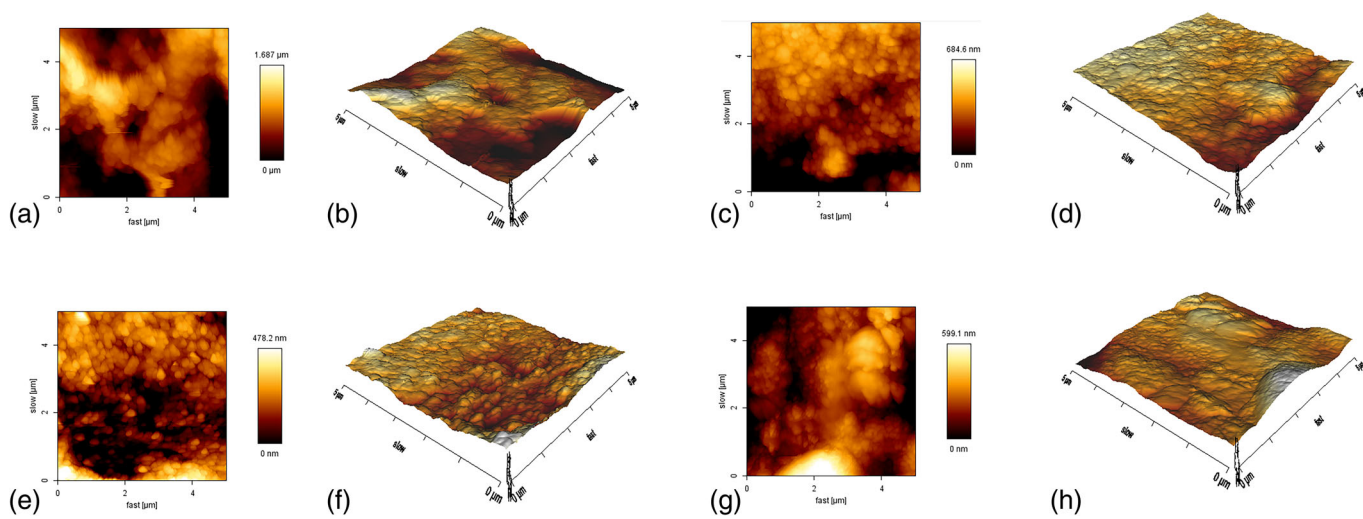


FIGURE 3 Atomic force micrographs of (a, b) $5 \times 5 \mu\text{m}^2$ area treated with Curodont, (c, d) treated with Curodont + toothpaste, (e, f) treated with Curodont + CPP-ACP, (g, h) treated with Curodont + BioMin F

indicated that the $\text{Ca}/\text{PO}_4^{3-}$ wt% ratio in Group 2 was significantly lower than in the other groups. In one study, the researchers reported a lower $\text{Ca}/\text{PO}_4^{3-}$ wt% ratio in P₁₁-4 compared to CPP-ACP, which partially agreed with our findings (Sindhura et al., 2018). This might be related to mineral loss with the use of acid to etch the enamel or the components of Curodont. The other materials increased the $\text{Ca}/\text{PO}_4^{3-}$ wt% ratio compared to the demineralization phase because of the presence and release of different amounts of Ca and PO_4^{3-} (Sindhura et al., 2018).

We used FESEM and AFM to identify the surface morphology and changes after the interventions. Our FESEM images of demineralized enamel showed the presence of porosities and spaces in the enamel surface due to dissolved minerals, which was in agreement with previous studies (Ceci et al., 2016; Kamal et al., 2020; Sindhura et al., 2018). The remineralizing agents may diffuse via the created spaces with remineralization occurring. FESEM images from Group 2 showed crystal-like structures that might be related to mineral diffusion and precipitation from the remineralization solution, which led to a reduction in pore volume space as reported in previous studies (Jablonski-Momeni & Heinzel-Gutenbrunner, 2014; Sindhura et al., 2018). However, we noted that enamel from the CPP-ACP group had increased deposition and globular arrangement of the minerals, which was also reported in another study (Sindhura et al., 2018). The application of BioMin F toothpaste caused crystal formation, which supported the results of prior studies (da Cruz, Hill, Chen, & Gillam, 2018; Hartgerink et al., 2001). AFM images showed that application of CPP-ACP and BioMin F led to decreased enamel roughness compared to P₁₁-4 alone or toothpaste.

One limitation of the current study was the difficulty with simulation of oral conditions and factors that influence early caries lesions in the laboratory setting, because of variations in biofilm and salivary secretion among patients. However, we maintained the teeth in artificial saliva and accurately applied the materials in accordance with the manufacturer's instructions. Other limitations include the lack of studies that have assessed the efficacy of some of these remineralizing agents in primary teeth. For this reason, we compared our results with studies performed on permanent teeth. In addition, primary teeth are more porous than permanent teeth and this may result in faster progression of lesions in the in vitro and in vivo studies.

Based on the results of this study, especially the effectiveness of P₁₁-4 associated with CPP-ACP, we recommend longer duration in vitro studies with more sample sizes and in vivo researches. Additional studies should be conducted that assess molecular dynamic simulations in order to determine if there are any affinities to different ions in the materials.

5 | CONCLUSION

The materials used in the present study increased EMH more than in the control group. The application of self-assembling peptide P₁₁-4 in combination with CPP-ACP was more effective than the other intervention groups in terms of improved EMH recovery. Other effective materials include fluoride bioactive glass toothpaste and CPP-ACP,

followed by fluoride toothpaste. The EDS results showed no significant differences between the groups in terms of the $\text{Ca}/\text{PO}_4^{3-}$ wt% ratio, except for P₁₁-4 which had a lower ratio. FESEM and AFM images showed that the CPP-ACP groups and fluoride bioactive glass toothpaste enhanced mineral deposition and reduced enamel roughness more than the toothpaste groups.

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ETHICS STATEMENT

The research protocol was approved by the Human Ethics Review Committee of the School of Dentistry, Shiraz University of Medical Sciences.

DATA AVAILABILITY STATEMENT

The datasets and images are available from the corresponding author.

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REFERENCES

- Alhussain, A. M., Alhaddad, A. A., Ghazwi, M. M., & Farooq, I. (2018). Remineralization of artificial carious lesions using a novel fluoride incorporated bioactive glass dentifrice. *Dental and Medical Problems*, 55, 379–382. <https://doi.org/10.17219/dmp/97311>
- Alkilzy, M., Tarabai, A., Santamaria, R. M., & Splieth, C. H. (2018). Self-assembling peptide P11-4 and fluoride for regenerating enamel. *Journal of Dental Research*, 97, 148–154. <https://doi.org/10.1177/0022034517730531>
- Bakry, A. S., Abbassy, M. A., Alharkan, H. F., Basuhail, S., Al-Ghamdi, K., & Hill, R. (2018). A novel fluoride containing bioactive glass paste is capable of re-mineralizing early caries lesions. *Materials*, 6(11), 1636. <https://doi.org/10.3390/ma11091636>
- Brauer, D. S., Karpukhina, N., O'Donnell, M. D., Law, R. V., & Hill, R. G. (2010). Fluoride-containing bioactive glasses: Effect of glass design and structure on degradation, pH and apatite formation in simulated body fluid. *Acta Biomaterialia*, 6, 3275–3282. <https://doi.org/10.1016/j.actbio.2010.01.043>
- Brunton, P. A., Davies, R. P., Burke, J. L., Smith, A., Aggeli, A., Brookes, S. J., & Kirkham, J. (2013). Treatment of early caries lesions using biomimetic self-assembling peptides—A clinical safety trial. *British Dental Journal*, 215, E6. <https://doi.org/10.1038/sj.bdj.2013.741>
- Ceci, M., Mirando, M., Beltrami, R., Chiesa, M., Colombo, M., & Poggio, C. (2016). Effect of self-assembling peptide P₁₁-4 on enamel erosion: AFM and SEM studies. *Scanning*, 38, 344–351. <https://doi.org/10.1002/sca.21276>
- da Cruz, L. P. D., Hill, R. G., Chen, X., & Gillam, D. G. (2018). Dentine tubule occlusion by novel bioactive glass-based toothpastes. *International Journal of Dentistry*, 4, 5701638. <https://doi.org/10.1155/2018/5701638>
- Dai, Z., Liu, M., Ma, Y., Cao, L., Xu, H. H. K., Zhang, K., & Bai, Y. (2019). Effects of fluoride and calcium phosphate materials on remineralization of mild and severe white spot lesions. *BioMed Research International*, 16, 1271523. <https://doi.org/10.1155/2019/1271523>

- Featherstone, J. D. (2008). Dental caries: A dynamic disease process. *Australian Dental Journal*, 53, 286–291. <https://doi.org/10.1111/j.1834-7819.2008.00064.x>
- Hartgerink, J. D., Beniash, E., & Stupp, S. I. (2001). Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science*, 23(294), 1684–1688. <https://doi.org/10.1126/science.1063187>
- Hojabri, N., Kaisarly, D., & Kunzelmann, K. H. (2020). Adhesion and whitening effects of P11-4 self-assembling peptide and HAP suspension on bovine enamel. *Clinical Oral Investigations*, 25, 3237–3247. <https://doi.org/10.1007/s00784-020-03654-1>
- Jablonski-Momeni, A., & Heinzel-Gutenbrunner, M. (2014). Efficacy of the self-assembling peptide P11-4 in constructing a remineralization scaffold on artificially-induced enamel lesions on smooth surfaces. *Journal of Orofacial Orthopedics*, 75, 175–190. <https://doi.org/10.1007/s00056-014-0211-2>
- Jablonski-Momeni, A., Nothelfer, R., Morawietz, M., Kiesow, A., & Korbmacher-Steiner, H. (2020). Impact of self-assembling peptides in remineralisation of artificial early enamel lesions adjacent to orthodontic brackets. *Scientific Reports*, 15(10), 15132. <https://doi.org/10.1038/s41598-020-72185-2>
- Kamal, D., Hassanein, H., Elkassas, D., & Hamza, H. (2020). Complementary remineralizing effect of self-assembling peptide (P11-4) with CPP-ACPF or fluoride: An in vitro study. *Journal of Clinical and Experimental Dentistry*, 1(12), e161–e168. <https://doi.org/10.4317/jced.56295>
- Kind, L., Stevanovic, S., Wuttig, S., Wimberger, S., Hofer, J., Müller, B., & Pieves, U. (2017). Biomimetic remineralization of carious lesions by self-assembling peptide. *Journal of Dental Research*, 96, 790–797. <https://doi.org/10.1177/0022034517698419>
- Kirkham, J., Firth, A., Vernals, D., Boden, N., Robinson, C., Shore, R. C., ... Aggeli, A. (2007). Self-assembling peptide scaffolds promote enamel remineralization. *Journal of Dental Research*, 86, 426–430. <https://doi.org/10.1177/154405910708600507>
- Krishnan, V., & Lakshmi, T. (2013). Bioglass: A novel biocompatible innovation. *Journal of Advanced Pharmaceutical Technology & Research*, 4, 78–83. <https://doi.org/10.4103/2231-4040.111523>
- Mehta, R., Nandalal, B., & Prashanth, S. (2013). Comparative evaluation of remineralization potential of casein phosphopeptide-amorphous calcium phosphate and casein phosphopeptide-amorphous calcium phosphate fluoride on artificial enamel white spot lesion: An in vitro light fluorescence study. *Indian Journal of Dental Research*, 24, 681–689. <https://doi.org/10.4103/0970-9290.127610>
- Oliveira, P. R. A., Barboza, C. M., Barreto, L. S. D. C., & Tostes, M. A. (2020). Effect of CPP-ACP on remineralization of artificial caries-like lesion: An in situ study. *Brazilian Oral Research*, 24(34), e061. <https://doi.org/10.1590/1807-3107bor-2020.vol34.0061>
- Patil, N., Choudhari, S., Kulkarni, S., & Joshi, S. R. (2013). Comparative evaluation of remineralizing potential of three agents on artificially demineralized human enamel: An in vitro study. *Journal of Conservative Dentistry*, 16, 116–120. <https://doi.org/10.4103/0972-0707.108185>
- Philip, N. (2019). State of the art enamel remineralization systems: The next frontier in caries management. *Caries Research*, 53, 284–295. <https://doi.org/10.1159/000493031>
- Schmidlin, P., Zobrist, K., Attin, T., & Wegehaupt, F. (2016). In vitro re-hardening of artificial enamel caries lesions using enamel matrix proteins or self-assembling peptides. *Journal of Applied Oral Science*, 24, 31–36. <https://doi.org/10.1590/1678-775720150352>
- Schwendicke, F., Splieth, C., Breschi, L., Banerjee, A., Fontana, M., Paris, S., ... Manton, D. J. (2019). When to intervene in the caries process? An expert Delphi consensus statement. *Clinical Oral Investigations*, 23, 3691–3703. <https://doi.org/10.1007/s00784-019-03058-w>
- Shen, P., McKeever, A., Walker, G. D., Yuan, Y., Reynolds, C., Fernando, J. R., ... Reynolds, E. C. (2020). Remineralization and fluoride uptake of white spot lesions under dental varnishes. *Australian Dental Journal*, 65, 278–285. <https://doi.org/10.1111/adj.12787>
- Sindhura, V., Uloopi, K. S., Vinay, C., & Chandrasekhar, R. (2018). Evaluation of enamel remineralizing potential of self-assembling peptide P11-4 on artificially induced enamel lesions in vitro. *Journal of the Indian Society of Pedodontics and Preventive Dentistry*, 36, 352–356. https://doi.org/10.4103/JISPPD.JISPPD_255_18
- Taha, A. A., Patel, M. P., Hill, R. G., & Fleming, P. S. (2017). The effect of bioactive glasses on enamel remineralization: A systematic review. *Journal of Dentistry*, 67, 9–17. <https://doi.org/10.1016/j.jdent.2017.09.007>
- Üstün, N., & Aktören, O. (2019). Analysis of efficacy of the self-assembling peptide-based remineralization agent on artificial enamel lesions. *Microscopy Research and Technique*, 82, 1065–1072. <https://doi.org/10.1002/jemt.23254>
- Vyavhare, S., Sharma, D. S., & Kulkarni, V. K. (2015). Effect of three different pastes on remineralization of initial enamel lesion: An in vitro study. *Journal of Clinical Pediatric Dentistry*, 39, 149–160. <https://doi.org/10.17796/jcpd.39.2.yn2r54nw24i03741>
- Wierichs, R. J., Kogel, J., Lausch, J., Esteves-Oliveira, M., & Meyer-Lueckel, H. (2017). Effects of self-assembling peptide P11-4, fluorides, and caries infiltration on artificial enamel caries lesions in vitro. *Caries Research*, 51, 451–459. <https://doi.org/10.1159/000477215>
- Zhang, Q., Zou, J., Yang, R., & Zhou, X. (2011). Remineralization effects of casein phosphopeptide-amorphous calcium phosphate crème on artificial early enamel lesions of primary teeth. *International Journal of Paediatric Dentistry*, 21, 374–381. <https://doi.org/10.1111/j.1365-263X.2011.01135.x>

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