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Remineralization potential of P11-4 and fluoride on secondary carious primary enamel: A quantitative evaluation using microcomputed tomography

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Abstract
The aim of this study was to assess the ability of self-assembling peptide (P11-4) diffusion, assembly, and remineralization to effect artificial secondary caries-like lesions in human primary teeth in vitro. Enamel-dentin blocks obtained from extracted human primary molars were embedded into epoxy resin blocks. Cavities (approximately 1 × 1 × 2 mm) were prepared on the surface using a high-speed diamond bur under constant water cooling and filled with composite restorative material (Filtek Z250; 3 M ESPE). The samples were immersed in demineralizing solution (20 ml) for 96 h to produce secondary caries lesions and divided into two groups according to the testing materials: fluoride varnish (Duraphat; Colgate, UK) and P11-4 (Curodont Repair; Credentis, Switzerland). Except for the control areas, all samples were remineralized for 3–5 min using the remineralizing agents, and then all the sections were placed in a pH-cycling system for 5 days at 35°C. The pH cycling procedure was followed by micro-CT analysis for the qualitative evaluation of surface changes. The Mann–Whitney *U* test was used to compare two independent groups. In the comparison of more than two dependent groups, Bonferroni smoothed pairwise analyses were used to determine the source of the Kruskal–Wallis *H* test difference. The results of the study revealed that the remineralization depths of the peptide group were higher than those of the fluoride group ($p < .01$). There was a statistically significant difference in remineralization effects between the fluoride and peptide groups. P11-4 can be considered as an effective remineralizing agent for secondary caries lesions.

KEYWORDS
P11-4, primary teeth, remineralization, secondary caries

Research Highlights

- Bone mineral density (BMD) scores of remineralization areas showed a statistically significant difference between fluoride and peptide groups ($p < .01$).
- The results of the study revealed that the BMD scores of the peptide group were higher than those of the fluoride group ($p < .01$).
- Curodont Repair may be an alternative treatment agent for remineralization of secondary carious lesions of primary teeth.

1 | INTRODUCTION

Dental caries is a common disease that remains a worldwide public health problem affecting adults and children (Chisini et al., 2018). It is characterized by demineralization of the inorganic structure and destruction of the organic portion of the tooth. The main etiologic factors of dental caries include cariogenic microorganisms, fermentable carbohydrates, a susceptible tooth, and time. However, in children, the microbial flora and immune system are not yet sufficiently developed, and newly erupted tooth surfaces may show hypoplastic defects. It is thought that there may be specific risk factors for dental caries in children (Harris et al., 2004).

In the oral cavity, the balanced processes of demineralization and remineralization occur regularly. However, diet differences, oral hygiene habit changes, or microbial activity can cause demineralization by disrupting the balance (Soares et al., 2017). Calcium and phosphate minerals, which make up most of the enamel, are dissolved due to the acidic pH, and pores are formed between the crystallites during the demineralization process. Remineralization is promoted by redepositing calcium and phosphate ions on the enamel and forming new crystallites. Therefore, the key to the prevention of dental caries is the regulation of the demineralization and remineralization equilibrium (Kind et al., 2017).

Dentists have most commonly performed dental restorations or replacements. In general, in dental practice, secondary caries is one of the main reasons for restoration replacement and occurs primarily in high-risk caries patients (Mjör, 2005). Unfortunately, a greater amount of dental tissue is lost in the treatment of secondary caries. This situation can eventually develop into a condition called the re-restoration cycle, which can lead to premature tooth loss (Nedeljkovic et al., 2015). In addition, secondary caries treatment results in enormous economic costs every year (Amend et al., 2018).

Until recently, the conventional treatment approach for dental carious lesions consisted of the removal of caries and restoration with a suitable restorative material. However, minimally invasive approaches in diagnosing and treating carious lesions have great importance, with decades of research in dentistry (Gomez, 2015). Contemporary treatment approaches used in pediatric dentistry for the remineralization of caries lesions promote the redeposition of calcium and phosphate ions and inhibit the demineralization of hydroxyapatite crystals (Üstün & Aktören, 2019).

Fluoride is highly effective in caries prevention, and the global decline in caries is mainly attributed to its use. The topical application of sodium fluoride varnishes has frequently been used in children to provide remineralization of carious lesions as a conventional approach (Üstün & Aktören, 2019). The cariostatic ability of fluoride has been recognized as the main reason for managing caries activity (Soares et al., 2017). Fluoride can integrate calcium fluoride compounds into the crystal structure and reduce the solubility of enamel (ten Cate, 1997). The potential protective effect of fluoride is restricted to nearly 30 μm on the outer enamel surface (Schmidlin et al., 2016). However, a regenerative approach has been suggested to aim to regenerate hydroxyapatite crystals within subsurface carious lesions using the essential remineralization capability of saliva (Kind et al., 2017).

Self-assembling peptide (P11-4) is an alternative procedure to the present approaches for managing carious lesions (Aggeli et al., 2001). P11-4 diffuses into the subsurface of carious lesions and forms a 3D matrix within demineralized tooth tissue, which contributes to de novo hydroxyapatite crystal formation in a process of biomimetic mineralization (Alkilzy et al., 2018). The formed peptide P11-4 matrix has a high affinity for calcium ions in saliva and enables enamel regeneration. Previous in vivo and in vitro studies have shown that P11-4 promotes subsurface mineralization in the presence of saliva (Brunton et al., 2013; Kirkham et al., 2007).

The aim of the present study was to evaluate the remineralizing efficacy on secondary caries lesions in primary teeth induced by the self-assembling peptide P11-4 and fluoride.

2 | MATERIALS AND METHODS

Power analysis was performed based on previous studies, and the minimum sample size for each group was determined to be seven to obtain 80% power at the 95% confidence level and the $\alpha = 0.05$ significance level. It was decided that the number of samples should be $n = 10$ to strengthen the results of the study.

2.1 | Sample preparation

Approval for this study (54022451) was obtained from the Bezmialem Vakif University Rectorate Clinical Research Ethics Committee. Twenty extracted caries-free human primary molars without cracks or erosion were cleaned and stored in physiological saline. Roots were removed at the cementoenamel junction with a water-cooled diamond disc. After that, crowns were cut in half in the corono–incisal direction with a long-speed diamond saw, resulting in 20 enamel/dentine blocks with rectangular form (approximately $2 \times 2 \times 5$ mm) shaped from the buccal surfaces of each crown under water cooling.

Specimens were embedded in epoxy resin blocks. Specimen surfaces were ground with flat grit and polished. Rectangular cavities (approximately $1 \times 1 \times 2$ mm) were prepared on the surface (in the center of the surface) using a high-speed diamond bur under constant water cooling. Cavities were filled with composite restorative material (Filtek Z250;3 M ESPE), followed by surface finishing and polishing. For the negative control group, half of the sample surfaces were covered with two coats of acid-resistant nail polish before the artificial caries process (Figure 1).

2.2 | Artificial secondary caries formation

All the samples were immersed in demineralizing solution (20 ml) for 96 h to produce secondary caries lesions. The demineralizing solution contained 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, and 0.05 M acetic acid;

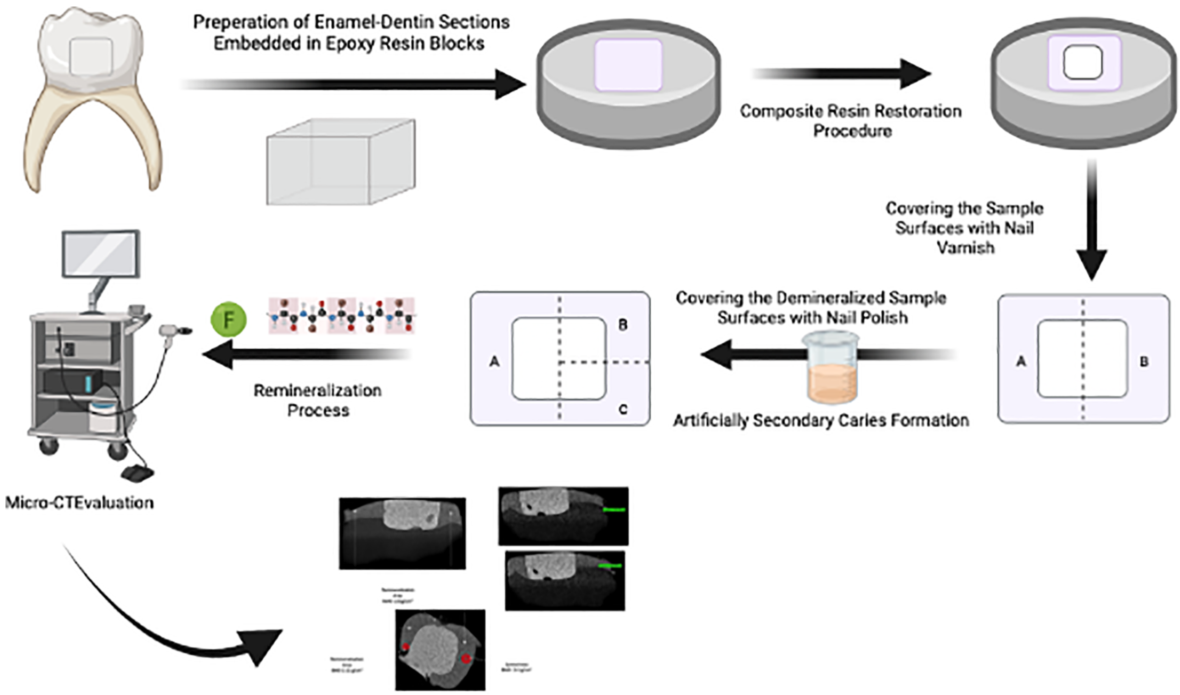

FIGURE 1 Schematic drawing of specimen preparation and testing

the pH was adjusted with 1 M KOH to 4.4 (Pinto et al., 2010; Sahin & Oznurhan, 2020). After demineralization, the samples were washed with deionized water. Then, half one of the restored demineralized enamel surfaces (the surface outside the negative control area) was coated with acid-resistant nail varnish (positive control surface), and samples were divided according to the tested materials:

1. Fluoride varnish (Duraphat Varnish; Colgate, UK)
2. P11-4 (Curodont Repair; Credentis, Switzerland)

2.3 | pH cycling procedure

Artificial saliva was prepared by mixing 2.4 g KCl, 1.7 g NaCl, and 0.1 g $MgCl_2$. $6H_2O$, 0.2 g $CaCl_2$. 2 H_2O, 0.2 g KSCH, 0.7 g KH_2PO_4, and 0.1 g H_3BO_3 with distilled water to an end volume of 1 L.

The demineralized areas, except the nail varnish-coated control surfaces on the samples, were remineralized for 3–5 min using the testing agents. After treatment with the respective remineralizing agents, the surfaces were then washed off with deionized water. All the samples were placed in the pH-cycling system for 5 days at 35°C (Robinson et al., 1992). Each 24-h period included 3 × 20 min exposures to demineralizing solution: 1.5 mM $Ca(NO_3)_2$, 0.9 mM KH_2PO_4, 50 mM acetic acid, pH 4.8. The content of the remineralizing solution was 1.5 mM $Ca(NO_3)_2$, 0.9 mM KH_2PO_4, 130 mM KCl, and 60 mM Tris, pH 7.4. The degrees of saturation (DSs) with respect to hydroxyapatite for each solution (calculated according to a previously published algorithm Shellis, 1988) were 0.29 and 14.06.

The pH cycling procedure was followed by micro-CT analysis for qualitative evaluation of surface changes.

2.4 | Micro-CT analysis

Micro-CT scans were performed with each sample in the same position with the standard scanning procedure to ensure that the X-ray was perpendicular to the sample surface. Analysis of 20 primary tooth samples was performed in a Skyscan 1172 micro-CT device at 89 kV–112 μA with 0.7 rotational steps, a random movement value of 20 and an average frame of 5 using an Al + Cu filter at 1 K. After the evaluations of the samples were made in the CTAn program, they were converted to jpeg format, and 3D and bone mineral density (BMD) analysis results were obtained with the section photos. BMD measurements of the control, demineralization and remineralization areas of the samples were performed on the 3D images in the same sections (Figure 2).

2.5 | Statistical analysis

All data were analyzed with SPSS 22.0 software for Windows (IBM Corp., Chicago, IL). The Shapiro–Wilk test was used to identify the normality of the data. The Mann–Whitney *U* test was used to compare two independent groups. The Kruskal–Wallis *H* test with Bonferroni correction was used for multiple comparisons. Data are shown as the medians and quarterly deficits (interquantile ranges). Eta squared interpretation (η^2) was used for the effect size of the

Kruskal–Wallis *H* test. The effect size of the Mann–Whitney *U* test was calculated with $r = Z/\sqrt{N}$. The level of significance was set at .05.

3 | RESULTS

There was a statistically significant difference between the control, demineralization, and remineralization depths in the fluoride varnish and P11-4 groups ($p < .05$). BMD scores of demineralized areas were significantly lower than remineralized areas in both groups; also, BMD scores of demineralization and remineralization areas were found to be significantly lower than the control area (Table 1).

BMD scores of control and demineralization areas did not show a statistically significant difference between fluoride and peptide groups ($p > .05$). However, BMD scores of remineralization areas were found to show a statistically significant difference between the two groups ($p < .01$). When the median values were examined, the remineralization BMD scores of the fluoride varnish group were found to be lower than those of the P11-4 group (Table 2).

The difference between BMD scores of demineralization and remineralization areas within each group was measured, and no statistically significant difference was found in the comparison between groups ($p > .05$) (Table 3).

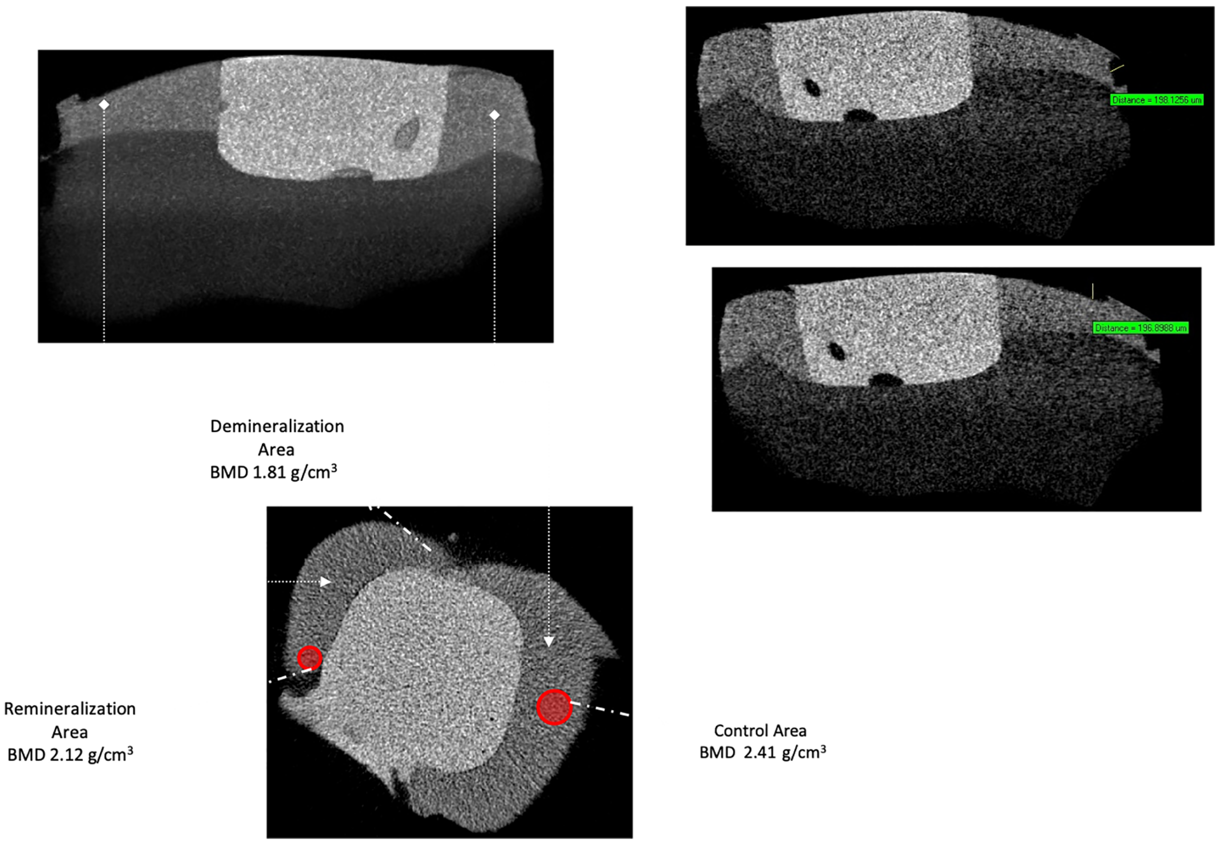

FIGURE 2 Micro-CT images of bone mineral density (BMD) analysis results of a sample from the P11-4 group

TABLE 1 Intergroup comparison for P11-4 and fluoride varnish

		Median (interquartile range) BMD scores	p^a	Pairwise	p^a
Fluoride Varnish	Control	2.4 (2.3–2.5)	.01	Demineralization < Remineralization	.03
	Demineralization	1.4 (1.3–1.5)		Demineralization < Control	.01
	Remineralization	1.9 (1.8–2.0)		Remineralization < Control	.04
P11-4	Control	2.4 (2.4–2.5)	.01	Demineralization < Remineralization	.03
	Demineralization	1.5 (1.5–1.8)		Demineralization < Control	.01
	Remineralization	2.2 (2.2–2.3)		Remineralization < Control	.03

Abbreviation: BMD, bone mineral density.
[a]Kruskal–Wallis test with Bonferroni Correction was used for statistical analysis.

TABLE 2 Comparison of groups according to fluoride varnish and P11-4

	Fluoride varnish group	P11-4 group	
	Median (interquartile range) BMD scores (g/mm^3)	Median (interquartile range) BMD scores (g/mm^3)	p^a
Control	2.4 (2.3–2.5)	2.4 (2.4–2.5)	.22
Demineralization	1.4 (1.3–1.5)	1.5 (1.5–1.8)	.14
Remineralization	1.9 (1.8–2.0)	2.2 (2.2–2.3)	.01

Abbreviation: BMD, bone mineral density.
[a]Mann–Whitney *U* test was used for statistical analysis.

TABLE 3 Findings related to comparison of demineralization-remineralization difference according to fluoride varnish and P11-4

	Fluoride varnish group	P11-4 group	
	Median (interquartile range) BMD scores (g/mm^3)	Median (interquartile range) BMD scores (g/mm^3)	p^a
Demineralization–remineralization difference	−0.48 [(−0.69)–(0.37)]	−0.64 [(−0.87)–(0.46)]	.11

Abbreviation: BMD, bone mineral density.
[a]Mann–Whitney *U* test was used for statistical analysis.

4 | DISCUSSION

Conventionally, the treatment of carious lesions consists of the removal of caries and replacement with a suitable restorative material. However, bioactive materials promoting a specific biological response when in contact with dental tissues have been defined. In recent years, biomimetic remineralization of dental caries has become important, and the results of our study also showed that P11-4 is an effective alternative biomimetic remineralization material to fluoride in secondary caries of primary teeth.

Kirkham et al. (2007) reported an effect for P11-4 on the de- and remineralization behaviors of caries-like lesions under in vitro conditions. Treatment with P11-4 resulted in a significant net mineral gain by the caries-like lesions after 5 days of pH cycling when compared with the control group. The pH cycling protocol requires that enamel and dentin be subjected to a series of demineralization and remineralization and is designed to mimic the dynamics of mineral loss and gain involved in caries formation (Buzalaf et al., 2010). The preferred pH cycling protocol for this study is based on the model described by Robinson et al. (1992). This pH cycling procedure provided basic quantitative data on the re- and demineralization behaviors of human enamel under oscillating conditions of pH, temperature, and mineral concentration similar to the oral environment (Robinson et al., 1992). Considering the results of the study by Kirkham et al. (2007), there is a significant decrease in demineralization depending on acid exposure during the pH cycle. The increase in remineralization in the peptide group seen in the results can be explained by both the low demineralization during the pH cycle and the stabilization of the mineral surfaces.

In the present study, micro-CT was used to evaluate the remineralizing efficacy of P11-4 and fluoride on demineralized human primary tooth enamel. Micro-CT has been used to measure mineral density changes in previous in vitro studies (Neves et al., 2019; Silvertown et al., 2017; Üstün & Aktören, 2019). This method has the advantages of being nondestructive and enabling high-resolution 3-dimensional analysis. Micro-CT enables analyzing the whole bulk of the specimen, visualizing the internal structure, and obtaining volumetric measurements due to its 3-dimensional nature.

In the present study, all tested remineralization agents were able to increase the mineral density in demineralized primary tooth enamel surfaces. To compare the remineralization efficacy of P11-4 on subsurface enamel caries lesions, we preferred a high-concentration fluoride-containing agent (22.600 ppm) in this study, and it was found that the effectiveness of P11-4 on the remineralization of artificial caries lesions was higher than that of fluoride varnish. An in vitro study (Bakry et al., 2018) evaluating the remineralization activities of BiominF and fluoride reported lower remineralization capacity in the fluoride group than in the BiominF group. These similar findings to our study may be explained by the high affinity of fluoride ions to combine with hydroxyapatite crystals to form fluorapatite in the superficial layers of demineralized enamel. This may have reduced the penetration of fluoride ions into the deeper layers of the subenamel surface lesion.

Alkilzy et al. (2018) investigated the safety and clinical effectiveness of P11-4 for the treatment of visible active early caries in permanent molars of pediatric patients in their clinical study. They reported that the combined use of P11-4 and fluoride had more effective remineralization capacity than the group in which fluoride was used alone.

Ideally, BMD analyzes should be performed separately at each part of the study. However, as a limitation of this study, micro-CT analysis was performed once at the end of the experiment, with all experimental groups placed on the same sample surface.

5 | CONCLUSION

Although there are many studies in the literature on the remineralization effects of P11-4 and other remineralizing agents, there are no in vitro studies comparing the remineralization effects of Curodont Repair and Duraphat varnish on primary teeth. According to our results, Curodont Repair is an alternative treatment agent for remineralization of secondary carious lesions of primary teeth.

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CONFLICT OF INTEREST

All authors contributed to this article, reviewed, and approved the current form of the article to be submitted. None of the authors have any financial interests to disclose regarding this work.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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