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Effect of Different Remineralization Agents on Artificial Caries Lesion: An in-vitro Study

Ecem Akbeyaz Şivet, Ayşe Nur Parlakyıldız Gökçe, Betül Kargül

Marmara University, Faculty of Dentistry, Department of Pediatric Dentistry, İstanbul, Türkiye.

Correspondence Author: Ecem Akbeyaz Şivet
E-mail: ecemakbeyaz@gmail.com
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ABSTRACT

Objective: Remineralization technologies have been shown to arrest or reverse early carious lesions. This study aimed to evaluate and compare the effect of different agents on enamel remineralization in-vitro.

Methods: Thirty-five enamel blocks were prepared and divided into the following groups: Group 1; CaGp and Xylitol-containing gel (R.O.C.S Medical Mineral Gel), Group 2; 1100 ppm NaF and 10% CPP-ACP toothpaste (MI Paste One), Group 3; 0.45% SnF2-1150 ppm F toothpaste (Enamelon), Group 4; Positive Control-1450 ppm NaF toothpaste (Colgate Total) and Group 5; Negative Control (deionized water). Microhardness was measured at baseline, after demineralization, and after respective treatments for different treatment groups using a digital Micro Vickers Hardness Tester. For producing demineralized lesions, samples were stored in acidic hydroxyethylcellulose (HEC, pH=4.8) for three days. The Paired Sample t-test, one-way ANOVA, and Tukey were used to compare data and SMH recovery (%SMHR) calculated among treatments.

Results: The mean baseline surface microhardness value was statistically non-significant between the groups (p=.378). CaGP and Xylitol-containing gel demonstrated having the most protective effect against demineralization. The surface remineralization potential of 1100 ppm NaF and 10% CPP-ACP containing novel toothpaste (MI Paste One) was almost similar to the positive control (1450 ppm Fluoride toothpaste) group (p>.05).

Conclusions: All treatment groups showed remineralization after respective treatments and these agents can be used as an effective preventive measure for pediatric patients.

Keywords: Remineralization, CaGP and Xylitol, Fluoride Toothpaste, Surface Microhardness, CPP-ACP

1. INTRODUCTION

Dental caries is a chronic multifactorial process and develops as a result of the combination of many genetic, diet, environmental, and lifestyle factors. Dental caries prevalence decrease has been declared however it remains one of the most common preventable non-communicable diseases worldwide. In recent years dental caries prevention has been the primary purpose of dental health care (1, 2).

The development of the caries process begins with the disruption of the balance between demineralization and remineralization (3). Minerals must be reabsorbed into carious areas to enhance remineralization (4). Enamel surface remineralization therapies can arrest the progression of early caries lesions. The minimally invasive dentistry approach focuses on the management of the condition that causes dental caries, restoring only when necessary, reducing the evolution of dental caries, and preventing the progression of caries lesions (5).

Recently developed remineralization technologies aim to prevent caries formation and treat at the initial term. Over the years, it has been generally accepted to recommend using fluoride-containing agents to prevent the demineralization of dental tissues (6). Regular toothbrushing with fluoridated toothpaste is the most common form of dental caries prevention (7). Sodium fluoride (NaF), a form of fluoride commonly used in oral health care products, significantly reduces the frequency of dental caries. SnF_2 (stannous fluoride), another form of fluoride, is used in dental care products and was indicated to have a suppressive effect against microbial biofilm by inhibiting the glycolysis of aciduric bacteria (8). Toothpaste containing SnF_2 has also proven effective in remineralizing early caries lesions (9).

Even though the remineralization effect of fluoride is generally considered successful, different enamel remineralization

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therapies have been suggested for personal and professional practice (10). Casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) is a bioactive agent, obtained from milk protein, and has been shown to support remineralizing efficacy of superficial enamel lesions in various studies (11, 12). CPP-ACP can enhance the activity of fluoride-based remineralization. Co-administration of CPP-ACP and fluoride has been demonstrated to have synergistic remineralizing action and antibacterial activity against cariogenic bacteria (13). MI Paste One (1100ppm NaF and 10% CPP-ACP toothpaste, GC America Inc, USA) is a toothpaste that combines the advantages of fluoride and CPP-ACP.

Calcium glycerophosphate (CaGP) and Xylitol-containing gel (R.O.C.S Medical Mineral Gel, DRC Group, Moscow, Russia) show remineralizing effect by increasing the mineral content on dental enamel has been reported (14). CaGP demonstrates a preventive action against demineralization by enhancing the resistance of hydroxyapatite crystals (15). Xylitol is a sugar alcohol and provides an alternative to sugar. It has been declared in the studies that the replacement of dietary sugar (sucrose) in chewing gums or sweets with Xylitol can decrease caries formation by increasing saliva production and reduction of cariogenic bacteria (16, 17).

Microhardness tests are easy, quick methods and are often used to investigate the physical structure of materials (18). Data from several studies have identified that enhanced remineralization is associated with increased enamel surface microhardness (19). Enhancing levels of microhardness of the enamel surface is dependent on the improvement of remineralization of the caries lesions has been stated (19). Featherstone et al. (20) investigated the artificial caries lesions by microhardness tests and found that mineral alterations in tooth structure as a result of remineralization and demineralization could be evaluated as microhardness change.

Many studies evaluated comparing CPP-ACP complex (21, 22) CaGP containing gel (15, 23) and conventional agents (24, 25) on caries remineralization with different results. Thus the main aim of this in vitro study is to demonstrate the remineralization activity of various agents and compare between groups using a digital Micro Vickers Hardness Tester.

Three null hypotheses were proposed: (1) the use of CaGP and Xylitol-containing gel will result in no significant difference in the enamel remineralization compared with 1450 ppm F toothpaste (positive control) (2) the use of CPP-ACP and 1100 ppm NaF containing toothpaste will result in no significant difference in the enamel remineralization compared with 1450 ppm F toothpaste (positive control) (3) the use of 0.45% SnF_2-1150 ppm F toothpaste will result in no significant difference in the enamel remineralization compared with 1450 ppm F toothpaste (positive control).

2. METHODS

2.1. Study Design

This study was approved by Marmara University, Faculty of Dentistry Ethics Committee (Approval number and date:2020-398/ 01.06.2020). The study was conducted following the Declaration of Helsinki.

The number of enamel samples in each group was evaluated as 7 specimens per group based on a previous study (G*power version 3.1.9.6, α=0.05, 1−β=0.80, effect size: 1.767) (26).

2.2. Preparation of Enamel Blocks

Twenty-eight freshly extracted permanent human molars were collected. Specimens were ultrasonically cleaned and removed from deposits/stains and sterilized following Occupational Safety and Health Administration recommendations (27). The infrared light transillumination (DIAGNOcam 2170U, Kavo, Biberach, Germany) was used to exclude the possibility of enamel cracks, decalcification of enamel, white spot lesions (WSLs), and extraction damage. After exclusion, the crowns of all teeth were cut from the roots at the cementoenamel junction and split mesiodistally into two halves using an ISOMET Low-Speed Saw cutting machine (Buehler, Lake Bluff, IL, USA).

The resultant 35 specimens were randomly distributed into five groups (n=7 specimens in each group) as follows: Group 1; CaGP+magnesium chloride ($MgCl_2$)+10% Xylitol (R.O.C.S. Medical Minerals Gel®), Group 2; 1100 ppm NaF and 10% CPP-ACP toothpaste (MI Paste One®), Group 3; 0.45% SnF_2-1150 ppm F toothpaste (Enamelon®), Group 4; Positive Control-1450 ppm NaF toothpaste (Colgate Total®), Group 5; Negative Control (deionized water). Group distribution and details are shown in Table 1. Enamel blocks (3 mm x 3 mm x 2 mm) were prepared from the labial surfaces. After embedding samples in epoxy resin, the superficial surface of the enamel was polished with water-cooled carborundum discs and 1200-grit waterproof silicon carbide paper (Amico), thereby removing about 200 µm of enamel (28).

2.3. Experimental Design

For producing demineralized lesions, samples were stored in acidic hydroxyethylcellulose (HEC, pH 4.8) for three days, following the procedure of Amaechi et al. (29). The solution was prepared by adding 100 mmol/l sodium hydroxide to 100 mmol/l lactic acids (pH value of 4.5). Following demineralization, surface microhardness measurements were made using the Vickers Hardness Number (VHN) indenter. The samples were immersed in one part agent and three parts artificial saliva (9 g: 27 ml) using a laboratory stand mixer until homogeneous. After the mixture was prepared, different remineralizing agents were applied to the enamel surface of each group with the applicator brush and left for 2 minutes (30, 31).

Artificial saliva was used in all treatment regimens and compositions were as follows: Magnesium chloride hexahydrate – ($MgCl_2 6H_2O$, 0.148 mmol/L), Dibasic potassium phosphate (K_2HPO_4, 4.59 mmol/L), Potassium phosphate monobasic (KH_2PO_4, 2.38 mmol/L) KCl (Potassium chloride, 8.39 mmol/l), calcium lactate (1.76 mmol/l), fluoride (0.05 ppm), sodium carboxymethyl cellulose (2.25 mmol/l), methyl-4-hydroxybenzoate ($HOC_6H_4CO_2CH_3$, 13.14 mmol/l). Solution pH was adjusted to 7.2 (32).

After each incubation, enamel specimens were carefully washed with sterile water to remove residual acid. The enamel specimens were then stored in filtered distilled water throughout the study. Surface microhardness was evaluated in 5 groups using the Vickers Hardness Test after treatment, and a comparative analysis was made.

Table 1. *Distribution of groups with respective active ingredients*

Groups	Agents	Active ingredients	Company
1	R.O.C.S (Remineralizing Oral Care Systems) Medical Minerals Gel®	Calcium glycerophosphate (CaGP) + Magnesium chloride (MgCl2) + 10% Xylitol	DRC Group, Moscow, Russia
2	MI Paste One®	1100ppm Sodium Fluoride (NaF) + 10% Casein phosphopeptides-amorphous calcium phosphate (CPP-ACP)	GC America Inc, USA
3	Enamelon® Fluoride Toothpaste	1150 ppm (0.45%) Stannous Fluoride (SnF_2) +Amorphous Calcium Phosphate (ACP)	Premier, USA
4	Positive Control (Colgate Total®)	1450 ppm (0.32%) Sodium Fluoride (NaF)	Colgate,USA
5	Negative Control	Deionized water	

2.4. Surface Microhardness Analysis

A digital Micro Vickers Hardness Tester (Wilson Wolpert Europe BV, 401 MVD, Netherland) fitted with a Vickers diamond and 200 Newton load was used to make indentations in the enamel surface. The loaded diamond was allowed to rest on the surface for 15 seconds at three different points, each 1 mm apart, and the mean value was saved as VHN.

The mean values of all three measurements of the midline surface at the three steps (baseline, after demineralization, after respective treatments). The percentage of surface microhardness recovery (%SMHR) was evaluated for each group using the following formula (33).

$$\%SMHR = 100 \times \frac{\text{SMH after remineralization} - \text{SMH after demineralization}}{\text{SMH baseline} - \text{SMH after demineralization}}$$

2.5. Statistical Analysis

Data is analyzed using SPSS 20.0 software. The Paired Sample t-test is used to compare surface microhardness before and after the treatments and the percent SMH recovery (%SMHR) calculated among treatments. One-way Analysis of variance (ANOVA) and Tukey was used for comparing data. The significant level (p) was set at .05.

3. RESULTS

A total of 50 specimens were initially demineralized, of which 35 specimens were selected for this study as described above. VHN for the baseline of all groups were compared, and there was no statistically significant difference found between groups (p=.378) (Table 2). After respective treatments, VHN remineralization of all the treatment groups had comparatively increased when compared to VHN demineralization (p< .05 for all groups) (Table 3).

Table 2. *Mean VHN for both groups measured at baseline*

	Remineralization agents	VHN baseline Mean±SD	p
Group 1	CaGP+Xylitol	374.91±5.65	.378
Group 2	CPP-ACP+1100 ppm NaF	373,91±6.55	
Group 3	1150 ppm SnF_2+ACP	363.70±8.92	
Group 4	1450 ppm NaF (positive control)	359.48±7.20	
Group5	Deionized water (negative control)	375.519±7.11	

VHN: Vickers Hardness Number, CaGP: Calcium glycerophosphate, CPP-ACP: Casein phosphopeptides-amorphous calcium phosphate, NaF: Sodium Fluoride, SnF_2: Stannous Fluoride, ACP: Amorphous Calcium Phosphate p<.05, One-way ANOVA*

Table 3. *The comparison of VHN after demineralization and remineralization of each treatment group*

	Groups	VHN demineralization	VHN remineralization	p
Group 1	CaGP+Xylitol	272.77±15.04	313.67±7.23	<.001*
Group 2	CPP-ACP +1100 ppm NaF	269.17±11.35	299.37±6.77	<.001*
Group 3	1150 ppm SnF_2+ ACP	242.49±8.04	267.81±5.36	<.001*
Group 4	1450 ppm NaF (Positive control)	252.40±6.40	279.95±5.74	<.001*
Group 5	Deionized water (Negative control)	259.06±6.44	271.89±5.51	<.002*

VHN: Vickers Hardness Number, CaGP: Calcium glycerophosphate, CPP-ACP: Casein phosphopeptides-amorphous calcium phosphate, NaF: Sodium Fluoride, SnF_2: Stannous Fluoride, ACP: Amorphous Calcium Phosphate
** p<.05, The Paired Sample t-test*

The mean %SMHR was found statistically different among groups (p<.001) (Table 4). The mean (±SD) %SMHR of groups were as follows: Group 1: 38.63 (±10.56), Group 2: 27.48 (±10.34), Group 3: 20.48 (±7.37), Group 4: 22.89 (±13.18) and Group 5: 11.35 (±7.38). The pairwise comparison of the percentage surface microhardness recovery (%SMHR) of treatment groups was shown in Table 5. There were significant differences between intergroup comparisons, but not between Group 2 (CPP-ACP+1100 ppm NaF) and Group 4 (1450 ppm NaF, positive control).

Table 4. *Mean and standard deviations (SD) of percentage surface microhardness recovery (%SMHR) for all groups*

	Microhardness Groups	SMHR (%) Mean±SD
Group 1	CaGP+Xylitol	38.63±10.56
Group 2	CPP-ACP+1100 ppm NaF	27.48±10.34
Group 3	1150 ppm SnF_2+ACP	20.48±7.37
Group 4	1450 ppm NaF (positive control)	22.89±13.18
Group 5	Deionized water (negative control)	11.35±7.38
p		<.001*

*CaGP: Calcium glycerophosphate, CPP-ACP: Casein phosphopeptides-amorphous calcium phosphate, NaF: Sodium Fluoride, SnF_2: Stannous Fluoride, ACP: Amorphous Calcium Phosphate, *p< .05, One-way ANOVA*

Table 5. *The pairwise comparison of percentage surface microhardness recovery (%SMHR) of treatment groups*

	Group 2 (CPP-ACP + 1100 ppm NaF)	Group 3 (1150 ppm SnF_2 + ACP)	Group 4 Positive control (1450 ppm NaF)	Group 5 Negative control (Deionized water)
Group 1 (CaGP and Xylitol)	.006*	< .001*	< .001*	< .001*
Group 2 (CPP-ACP+1100 ppm NaF)		< .001*	.95	< .001*
Group 3 (1150 ppm SnF_2+ACP)			< .001*	.030*
Group 4 Positive control (1450 ppm NaF)				< .001*

CaGP: Calcium glycerophosphate, CPP-ACP: Casein phosphopeptides-amorphous calcium phosphate,
*NaF: Sodium Fluoride, SnF_2: Stannous Fluoride, ACP: Amorphous Calcium Phosphate,*p< .05, ANOVA, Tukey HS*

The first hypothesis of the present study, (1) the use of CaGP and Xylitol-containing gel will result in no significant difference in the enamel remineralization compared with 1450 ppm F toothpaste, have to be rejected. Second null hypothesis (2) the use of CPP-ACP and 1100 ppm NaF containing toothpaste will result in no significant difference in the enamel remineralization compared with 1450 ppm F toothpaste, accepted. The third null hypothesis (3), the use of 0.45% SnF_2-1150 ppm F toothpaste will result in no significant difference in the enamel remineralization compared with 1450 ppm F toothpaste, has to be rejected.

4. DISCUSSION

Remineralizing agents can preserve caries emergence or intercept the worsening of enamel demineralization, and this could be provided by boosting calcium phosphate levels in the environment (28). In our study, we use demineralization and remineralization solutions to imitate the caries formation and anti-caries process (29,34). To produce demineralized lesions, we chose acidic hydroxyethylcellulose (HEC), and the pH was adjusted to 4.8. Specimens were stored in this solution for three days, based on the study protocol conducted by Amaechi et al. (29). Artificial saliva could promote remineralization on early enamel caries lesions, according to research by Huang et al. (35). In the present study, we used artificial saliva to prepare a respective remineralizing treatment to apply enamel sample surfaces. Microhardness changes are directly related to mineral alteration in the subsurface lesion (36). Kielbassa et al. (37) reported a significant association between microhardness and the mineral ratio of caries lesions. Surface microhardness values are a commonly used technique for assessing the level of mineralization and vary according to the level of calcium content in enamel specimens (38,39). We used the Vickers microhardness test in our study to evaluate demineralized and remineralized enamel samples since it has been used in many studies and is a rapid and non-damaging method (33, 39). Multiple variables might change the explication of surface microhardness values, such as the setting of samples, exposed angle, and the region picked. We considered these factors and measured each enamel midline surface at three different times. No statistically significant difference was observed in baseline surface microhardness values between all groups (p> .05).

Calcium glycerophosphate and Xylitol-containing agent showed remineralizing and antibacterial efficacy in some studies (15, 40). In the present study, Group 1 (CaGP and Xylitol-containing gel) showed the highest %SMHR. Sezer and Kargul declared that CaGP containing agent has remineralization potential on hypomineralized enamel in their in-vivo study, on the other hand when comparing with the casein phosphopeptide amorphous calcium fluoride phosphate (CPP-ACFP) there were no statistically significant differences observed (15). Another in-vitro study reported that there was no significant difference between the CPP-ACP, CPP-ACFP, and CaGP+MgCl2+Xylitol groups in terms of the amount of advancing in the %SMHR (23).

Stannous fluoride has a protective effect against dental plaque and dentin hypersensitivity (9). SnF_2-containing toothpaste has been shown more effective in reversing early carious lesions compared with conventional NaF-containing toothpaste (41). In the present study, SnF_2 toothpaste showed a lower %SMHR than the 1450 ppm F toothpaste group and of the CPP-ACP and 1100 ppm NaF group. Likewise our study results, another study reported SnF_2 toothpaste showed

inferior efficiency against 1100 ppm fluoridated toothpaste (42).

CPP-ACP has demonstrated its impact on the significant recovery of initial enamel lesions in-vivo and in-vitro (43, 44, 45). According to our study results, CPP-ACP and 1100 ppm NaF toothpaste (Group 2) showed higher remineralization against 1150 ppm SnF_2 (0.45%) + ACP (group 3) and negative control (Group 5). However, there was no significant difference between the 1450 ppm NaF toothpaste-positive control (Group 4). Similar to our results, Vashisht et al. (46) they were reported that CPP-ACP indicated significant remineralization against the control group (deionized water). CPP-ACP promotes the stabilization of calcium and phosphate ions and mineral deposition in an acidic environment for enhanced buffering. In some studies, CPP-ACP has shown superior efficacy to fluoride in caries management (38, 39). By contrast, in 2016 Rirattanapong et al. (47) reported that 500 ppm F toothpaste had a superior remineralization efficiency than CPP-APP.

Some researchers have shown that CPP-ACP 's remineralization capacity is equal to or lesser than fluoride (33, 48). In an in-vitro study, Gonçalves et al. in 2021 declared that CPP-ACP+F paste promoted a superior efficacy compared with 1100 ppm F toothpaste (21). We designed an in-vitro study; there were inadequate calcium and phosphate ions values to promote remineralization; thus, it could be a reason for the different results between studies. In the current study CPP-ACP and 1100 ppm NaF toothpaste (Group 2) did not show additional surface remineralization against positive control (Group 4). A possible explanation for these results may be; that CPP attaches to apatite crystal on the enamel surface and preserves ionic diffusion to allow minerals to penetrate deeper; thus, remineralization occurs in both the superficial layer and body of the lesion. Similar to our study results, Vyavhare et al. (49) found that the CPP-ACP group and NaF (1000 ppm) group had the same %SMHR, and there was no significant difference between them.

Our study limitations were; that in-vitro conditions could not simulate the complete demineralization/remineralization cycle in the oral cavity, and the artificial saliva and artificial caries lesions did not contain bacteria as in the actual environment. The results of this investigation show that CaGP+Xylitol has the best effectiveness in remineralizing enamel lesions. CPP-ACP and 1100 ppm NaF toothpaste showed similar results as the positive control group.

5. CONCLUSION

It could be considered that CaGp and Xylitol-containing gel and CPP-ACP and 1100 ppm NaF toothpaste can be used in preventive dentistry as an alternative to conventional fluoride toothpastes and a new approach to reducing the risk of caries. Further randomized controlled trials should be focused on different groups to confirm the accuracy of long-term effects, administration patterns, and dosage for these agents to be widely used in preventive and therapeutic applications.

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