Original Article

The Remineralization Effect of Calcium Glycerophosphate in Fluoride Mouth Rinse on Demineralized Primary Enamel: An *in vitro* Study

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ABSTRACT

Aim: To evaluate the remineralization effect of a fluoride mouth rinse containing calcium glycerophosphate in fluoride mouth rinse based on the surface microhardness of demineralized primary enamel. **Materials and Methods:** 40 sound primary incisors were placed into self-curing acrylic resin and subjected to a demineralizing solution for 5 days, resulting in the formation of artificial caries. The teeth were categorized into four groups ($n = 10$): group I artificial saliva, group II sodium fluoride, group III sodium fluoride + sodium monofluorophosphate, and group IV sodium monofluorophosphate + calcium glycerophosphate. The specimens received a pH cycling procedure and were submerged twice in their assigned groups for 7 days. The baseline, after demineralization, and after remineralization surface microhardness values were determined. One-way analysis of variance (ANOVA) was used to analyze the mean surface microhardness between groups and one-way repeated measures ANOVA for the mean surface microhardness within each group and Bonferroni's for multiple comparisons at 95% confidence level. The percentage recovery surface microhardness was determined by calculating the average surface microhardness. **Results:** After demineralization, the mean surface microhardness in all groups significantly decreased. After remineralization, group I had the lowest surface microhardness values and the percentage recovery surface microhardness (P value < 0.001), and group IV had the highest surface microhardness values and the percentage recovery surface microhardness (P value < 0.001). No significant difference was found between groups II and III (P value = 0.365). **Conclusions:** Fluoride mouth rinse containing calcium glycerophosphate has a remineralization effect on demineralized primary enamel.

Keywords: *Calcium glycerophosphate, caries, microhardness, primary teeth, remineralization*

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Introduction

Dental caries, a noncommunicable disease (NCD), is a preventable disease that has significant effects on an individual's quality of life.[1] It is caused by an imbalance in the oral environment that favors demineralization by acid produced by bacteria compared with remineralization by salivary components or therapeutic agents.[2,3] Recent studies conducted over the past few years have shown that the prevalence of dental caries remains high and is expected to persist in the future. These findings emphasize the need to establish and enhance promotion and preventive programs.[4,5]

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Fluoride mouth rinses have been widely used for many years to reduce the incidence of dental caries in children at high risk. Numerous studies have demonstrated that fluoride mouth rinses promote the remineralization of carious lesions and that frequent use of fluoride mouth rinses under supervision significantly reduces the incidence of carious lesions in children.[6] Fluoride ions are well-known for their ability to induce remineralization in enamel by forming fluorapatite, which is more resistant to acid dissolution compared with hydroxyapatite. Fluoroapatite is composed of 10 calcium ions and 6 phosphate ions for every 2 fluoride ions. Therefore, an abundance of calcium and phosphate ions is necessary to remineralize the enamel with fluorapatite.[2,7]

Previous studies have found that combining fluoride with other chemical agents enhances its efficiency, allowing it to more efficiently remineralize incipient caries.[8-10] Numerous studies have been performed to improve the calcium and phosphate content of dental products to develop new technologies for a greater remineralization effect by raising the calcium and phosphate content of the environment.[8-10]

Most mouth rinses used for dental tissue remineralization contain only fluoride or calcium because fluoride, the most electronegative element, easily combines with calcium to form CaF_2, which is insoluble in water. This undesirable interaction between calcium and fluoride decreases the bioavailability of calcium and fluoride in products containing both elements.[7,11,12] Therefore, a fluoride mouth rinse containing calcium may reduce fluoride's remineralization effect. To overcome this limitation, commercial fluoride mouth rinses containing calcium glycerophosphate have been developed.

Studies found that calcium glycerophosphate (CaGP) was a cariostatic agent. CaGP is an anticariogenic calcium phosphate salt made from organic calcium phosphate. CaGP is thought to facilitate plaque-pH buffering by increasing the calcium and phosphorus levels in plaque, as well as increasing the calcium and phosphate in the hydroxyapatite in enamel. Furthermore, CaGP has demonstrated antibacterial effects,[13,14] and it is used in toothpastes, usually in conjunction with sodium monofluorophosphate (SMFP).[13-17] Puig-Silla *et al.*[18] revealed that adding CaGP to SMFP mouth rinse increased the remineralization effect more than using fluoride mouth rinse alone. These studies suggested that adding CaGP improves remineralization without increasing the fluoride concentration, which can have deleterious effects on children.[13-18]

Most prior studies evaluating the efficacy of CaGP were conducted on bovine teeth, which can mimic permanent teeth. However, there is no report on the efficiency of CaGP on human primary teeth. The major differentiation between human primary and permanent teeth is that primary teeth possess a thinner enamel layer with a lower mineral composition, resulting in a greater diffusion coefficient and a greater susceptibility to acid dissolution, which may impact the tooth's ability to remineralize.[19] Thus, the remineralization effect may differ between primary and permanent teeth.

Although there have been several reports on the efficacy of CaGP on enamel remineralization,[13-18] there is no report on the effectiveness of a mouth rinse containing calcium glycerophosphate performed using demineralized primary enamel. The aim of this *in vitro* study was to evaluate the remineralization effect of a fluoride mouth rinse containing CaGP based on the surface microhardness of demineralized primary enamel. The null hypothesis was that fluoride mouth rinse containing CaGP has no remineralization effect on demineralized primary enamel.

Material and Methods

Setting and design

The study protocol was approved by the Ethics Committee of Mahidol University (COE.No.MU-DT/PY-IRB2021/027.2308).

The calculation of sample size was based on Rirattanapong *et al.*,[20] which evaluated the remineralization effect of a fluoride mouth rinse containing tricalcium phosphate based on the surface microhardness in primary teeth. This finding shows that the mean microhardness after 7 days of pH-cycling was 179.32 ± 25.59 VHN for the deionized water group, 231.85 ± 18.73 VHN for the 0.05% sodium fluoride plus tri-calcium phosphate (NaF plus TCP) group, and 225.10 ± 19.99 VHN for the 0.05% NaF group. The sample size calculation in this study was conducted using G*power program version 3.1.2 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, German) with one-way analysis of variance (ANOVA) with $\alpha = 0.05$ and $\beta = 0.10$. The calculation indicated that 10 teeth were required for each group.

Sampling criteria

40 sound human primary incisors were collected from teeth that were extracted or naturally exfoliated and kept in normal saline until used. Sound teeth were selected to be used in this study. The exclusion criteria were having cracks, fluorosis, enamel hypoplasia, white or brown lesions, or tetracycline-stained teeth. A bur (Cerinlay, Intensiv, Viganello, Switzerland) in a high-speed handpiece (J. Morita Manufacturing Corp.,

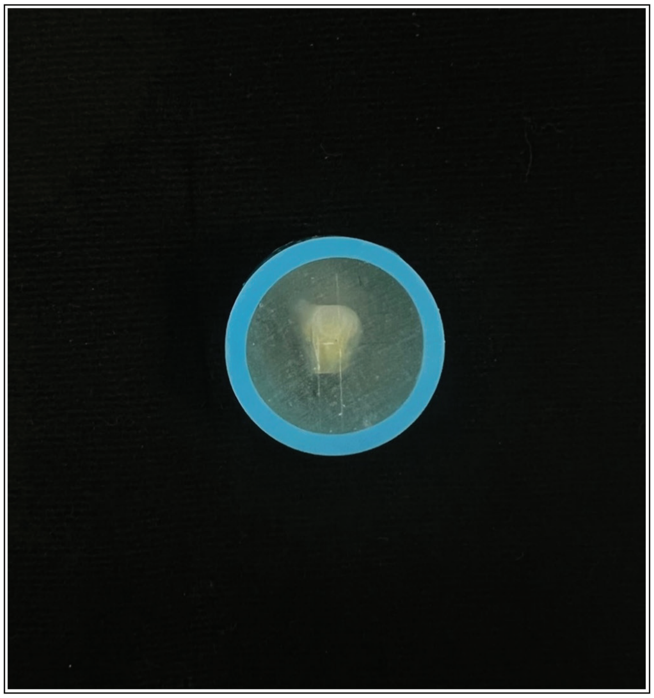

Figure 1: Specimen preparation

Kyoto, Japan) was used to remove the radicular portion of each tooth. Self-cured acrylic resin (Ortho-Acrylic P/L, Homedent Group Co., Ltd., Bangkok, Thailand) was used to embed the crown specimens. The labial surfaces were wet ground using 1000, 1500, 2000, and 2500 grit silicon carbide paper (Buehler, Lake Bluff, IL, USA) to achieve a smooth and flat surface. A scalpel (Cutter H-111, Nan Mee Co., Ltd, Bangkok, Thailand) was used to make cuts that demarcated a 2 × 2 mm test window. This procedure was conducted to aid in the proper alignment of the specimen during the surface microhardness test [Figure 1]. The baseline surface microhardness of the sound enamel was measured on the labial surface via a Vickers indenter (FM-700e Type D, Future-tech, Tokyo, Japan) applying a force of 100 g for a duration of 15 s.[21] During each experimental stage, a total of four indentations were conducted on each specimen. The mean surface microhardness was employed and determined as the percentage recovery of SMH (%SMHR) = 100 × (SMH after remineralization – SMH after demineralization)/(SMH at baseline – SMH after demineralization).[21]

Methodology

The present study employed two demineralization solutions and one remineralization solution. Demineralizing solution 1 (D1) comprised 2.2 mM $CaCl_2$ (Ajax Finechem Pvt., Ltd., New South Wales, Australia), 2.2 mM NaH_2PO_4 (Ajax Finechem Pvt., Ltd.), 0.05 M acetic acid (EMD Millicorp., MA, USA), and 1 M KOH (Ajax Finechem Pvt., Ltd.) to adjust the pH to 4.4. Demineralizing solution 2 (D2) was formulated with identical components as D1, with the exception of pH adjustment to 4.7. The remineralizing solution (R) was composed of 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.15 M KCl (Ajax Finechem Pvt., Ltd.), and 1 M KOH to achieve a pH of 7.0.[21] Each pH-cycling process used newly prepared demineralizing and remineralizing solutions, which were kept in separate containers for each group throughout the trial.

Using a modified version of Kasemkhun and Rirattanapong's method, the specimens were submerged for 5 days in D1 at 37°C in an incubator (Sheldon Manufacturing, model 1545, Cornelius, OR, USA) to create artificial caries. After rinsing the specimen with deionized water, it was patted dry using tissue paper. The specimens were placed within a solution of artificial saliva that included 0.65 g/L KCl, 0.058 g/L $MgCl_2$ (Ajax Finechem Pvt., Ltd.), 0.165 g/L $CaCl_2$, $K_2(HPO_4)_2$ (Ajax Finechem Pvt., Ltd.), and $KH_2(PO_4)_3$ (Ajax Finechem Pvt., Ltd.), 2 g/L $NaCO_2CH_3$ cellulose (Sigma-Aldrich Pvt., Ltd., Singapore), and deionized water until used.[21]

The specimens were pooled and randomly assigned into four groups of 10 teeth: group I artificial saliva (no treatment), group II sodium fluoride (NaF – 220 ppmF) (Listerine® total care zero alcohol; Johnson & Johnson, Thailand), group III sodium fluoride + sodium monofluorophosphate (NaF+SMFP – 241 ppmF) (Fluocaril® zero alcohol double mint mouthwash; Meiyume Manufacturing, Thailand), and group IV sodium monofluorophosphate + calcium glycerophosphate (SMFP + CaGP – 224 ppmF + 75 ppmCa) (Fluor Kin mouthwash; Industria Farmaceutica Andromaco, Mexico) [Table 1].

The specimens underwent a pH-cycling protocol, which involved a 3-h period of demineralization in the D2 solution, followed by a 2-h period of remineralization in the R solution. This was followed by another 3-h period of demineralization in the D2 solution. Finally, the specimens were immersed in the R solution overnight at a temperature of 37°C in an incubator. Prior to and following the overnight remineralization process, the specimens were submerged in the assigned test group solution for a duration of 1 min.[21]

Statistical analysis

SPSS version 26 (IBM Corp., Armonk, NY, USA) was employed to conduct a one-way repeated measures ANOVA to compare surface microhardness values at baseline, after demineralization, and after remineralization within the groups. Additionally, the

Table 1: Mouth rinses used in this study

Group	Active ingredients	Trade name	Other ingredients	Manufacturers
NaF	Sodium fluoride 220 ppmF	Listerine® total care zero alcohol	Water, sorbitol, propylene glycol, paloxamer407, sodium lauryl sulfate, flavor, eucalyptol, zinc chloride, benzoic acid, sodium benzoate, sodium saccharin, thymol, methyl salicylate, menthol, aroma, sucralose, Cl 16035, Cl 42090	Johnson & Johnson, Thailand
NaF + SMFP	Sodium monofluorophosphate sodium fluoride total 241 ppmF	Fluocaril® zero alcohol double mint mouthwash	Water, glycerin, sodium benzoate, PPG-26 buteth-26, PEG-40 hydrogenated castor oil, flavor, cetylpyridinium chloride, sodium saccharin, citric acid, Cl47005, Cl42051	Meiyume Manufacturing, Thailand
SMFP + CaGP	Sodium monofluorophosphate 224 ppmF calcium glycerophosphate 75 ppmCa	Fluor Kin mouthwash	Aqua, glycerin, sorbitol, propylene glycol, PEG-40 hydrogenated castor oil, xylitol, aroma, sodium methylparaben, citric acid, cetylpyridinium chloride, sodium propylparaben, sodium saccharin, potassium acesulfame, CI 14720	Industria Farmaceutica Andromaco, Mexico

Table 2: Micro hardness value at baseline, after demineralization, after remineralization, and the %SMHR

	Baseline	After demineralization	After remineralization	% SMHR	*P* value
No treatment	341.40 ± 5.65	213.37 ± 7.11	242.48 ± 6.91	22.82 ± 3.11	<0.001*
NaF	339.60 ± 7.66	217.98 ± 9.22	315.23 ± 3.81	80.22 ± 6.39	<0.001*
NaF + SMFP	338.16 ± 11.16	213.29 ± 7.05	307.82 ± 8.61	75.82 ± 6.11	<0.001*
SMFP + CaGP	344.66 ± 6.02	217.25 ± 4.91	335.94 ± 5.24	93.31 ± 3.93	<0.001*
P value	0.307	0.328	<0.001*	<0.001*	

Data are represented as mean ± SD; last row *P* value: one-way ANOVA, right column *P* value: repeated measure ANOVA; % SMHR: % recovery of surface microhardness after remineralization;

*Significant at the 0.05 level ($\alpha = 0.05$)

Table 3: Multiple comparison (*post hoc* test) of the microhardness in the different groups

	NaF	NaF + SMFP	SMFP + CaGP
After Remineralization			
No treatment	72.74* ($P < 0.001$)	65.34* ($P < 0.001$)	93.46* ($P < 0.001$)
NaF	–	7.40 ($P = 0.083$)	20.71* ($P < 0.001$)
NaF+SMFP	–	–	28.12* ($P < 0.001$)
% SMHR			
No treatment	57.40* ($P < 0.001$)	53.00* ($P < 0.001$)	70.49* ($P < 0.001$)
NaF	–	4.40 ($P = 0.365$)	13.10* ($P < 0.001$)
NaF+SMFP	–	–	17.49* ($P < 0.001$)

Multiple comparison was performed using the Bonferroni test

*The mean difference was significant at the 0.05 level ($\alpha = 0.05$)

differences in the surface microhardness values between the groups at each stage were determined using one-way ANOVA and the Bonferroni method for multiple comparisons with 95% confidence level.

Results

The mean SMH value at baseline was 340.95 ± 12.25 VHN with no significant difference among groups (*P* value = 0.307) [Table 2]. Furthermore, the mean SMH values after demineralization were 215.47 ± 3.94 VHN, which significantly decreased with a mean drop of 36.8% from baseline, however, the differences between the groups were not significant (*P* value = 0.328) [Table 2].

The mean SMH values and %SMHR in each group significantly increased after remineralization. The SMFP+CaGP group demonstrated the highest SMH values and %SMHR (*P* value < 0.001), and the no treatment group had the lowest SMH values and %SMHR (*P* value < 0.001) [Table 2]. There was no significant difference in SMH values and %SMHR between the NaF and NaF+SMFP groups (*P* value = 0.365) [Table 3].

Discussion

The present study evaluated the remineralization effect of a fluoride mouth rinse containing calcium glycerophosphate based on the surface microhardness of demineralized primary enamel. The mean baseline SMH value in our study was similar to the findings in Siripipat *et al.* and Kasemkhun and Rirattanapong.[21,22] After demineralization, the mean SMH was also similar to Siripipat and colleagues' study.[22] After remineralization, every group demonstrated a significant increase in mean microhardness and %SMHR. The increase in mean microhardness and %SMHR observed in the no-treatment group could be attributed to the remineralization properties of the artificial saliva used in this investigation.[23] For the mouth rinse groups, the findings were consistent with the hypothesis that the existence of fluoride and calcium facilitates the process of remineralization in demineralized lesions.[2,24]

Previous studies have demonstrated that fluoride-containing calcium-phosphate products are beneficial for remineralizing caries.[8-10] Additionally, many prior studies have reported that fluoride toothpaste containing CaGP effectively rehardened demineralized enamel.[15-17] Moreover, Puig-Silla *et al*.[18] revealed that fluoride mouth rinse containing CaGP had a markedly greater potential for remineralization compared with fluoride mouth rinse alone at the same fluoride ion concentration in bovine enamel. These findings are consistent with our results that a fluoride mouth rinse containing CaGP can remineralize the demineralized enamel of primary enamel. The remineralization of enamel subsurface lesions is related to the fluoride and calcium phosphate levels in the lesion environment. Remineralization occurs by the diffusion of calcium, phosphate, and fluoride ions into the lesion through pores in the enamel creating a CaF_2 surface layer.[2,12]

The remineralizing effect of CaGP in this study resulted from the CaGP acting directly on the enamel.[13] The CaGP molecule consists of an ionic bond between calcium and phosphate and a covalent bond between glycerol and phosphate.[25] CaGP has been shown to increase calcium and phosphate levels, which are essential for tooth mineralization.[2,13] This causes CaGP to have a direct effect on enamel and reduce the acid solubility of hydroxyapatite.[13] However, other possible mechanisms of action for CaGP have been proposed, including CaGP's ability to prevent plaque pH from dropping, reducing plaque mass, and elevating calcium and phosphorous levels.[13] Furthermore, CaGP and SMFP work together to create a synergistic effect. These compounds have a stronger cariostatic effect on teeth and help to prevent enamel dissolution.[13,15-17] However, previous studies also found that the addition of CaGP in fluoride varnish did not enhance the remineralization efficacy of fluoride.[26,27] This might be because of the calcium fluoride precipitation that reduces fluoride's remineralizing effect. However, our results indicated that the fluoride mouth rinse containing CaGP had a remineralization effect on demineralized primary enamel. This finding could be due to the different types of fluoride and calcium contained in the mouth rinse, as well as discrepancies in the experimental protocols employed in the investigations.

The finding of this study rejected the null hypothesis that fluoride mouth rinse containing CaGP have no remineralization effect on demineralized primary enamel. The observed remineralization effect appears to be a result of the synergistic interaction between CaGP and fluoride. This discovery potentially indicates that the use of fluoride mouth rinse containing CaGP could serve as an effective alternative for individuals who wish to enhance the remineralization process of their teeth. One limitation of this study is that despite the pH-cycling model used in this study being designed to mimic the oral cavity's demineralization and remineralization processes, as well as simulate caries development and anti-caries effects, it still cannot accurately mimic the complicated intraoral environment.[28] Further clinical investigation is needed to validate and evaluate the efficacy and efficiency of fluoride mouth rinse containing CaGP.

Conclusion

The present study showed that fluoride mouth rinse containing calcium glycerophosphate has a remineralization effect on demineralized primary enamel. The observed result can be attributed to the combined effects of CaGP and fluoride, which exhibit a synergistic effect. The finding of this study might suggest that fluoride mouth rinse containing CaGP could be an alternative option for patients seeking to promote the remineralization of their teeth.

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Conflicts of interest

Nil.

Authors contributions

PR and WP collaborated to formulate and design the research framework. The experiments were conducted by PT, and all writers contributed to the analysis of data and statistics. The manuscript was authored and subsequently edited by PT and PR. The final version of the manuscript was approved by all authors.

Ethical policy and institutional review board statement

This study was approved by the Ethics Committee of Mahidol University, Thailand (COE.No.MU-DT/PY-IRB2021/027.2308).

Patient declaration of consent

Nil.

Data availability statement

The additional data of this study are available on request from Dr. Pannaros Torsakul at pannaros.tor@gmail.com.

List of Abbreviations

CaGP: calcium glycerophosphate
NCD: noncommunicable disease
%SMHR: percentage recovery of SMH
NaF: sodium fluoride
SMFP: sodium monofluorophosphate
SMH: surface microhardness
TCP: tri-calcium phosphate

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