

EFFECT OF DIFFERENT REMINERALIZATION PROTOCOLS ON DEMINERALIZED ENAMEL SURFACE MICROHARDNESS - IN VITRO COMPARATIVE STUDY

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ABSTRACT

Objective: The purpose of this study was to evaluate the effect of different remineralization protocols: Fluoride mouth wash (Listerine total care zero), CPP-ACP, CPP-ACFP and CPP-ACP +Fluoride mouth wash on demineralized enamel surface micro hardness.

Materials and Methods: A total of thirty non carious freshly extracted human premolar orthodontic purpose were obtained as specimens and sections into two half were demineralized. The specimens were randomly divided into the following six treatment groups: (a): positive control, (b): negative control, (c): Listerine total care zero mouth wash, (d): CPP-ACP, (e): CPP-ACFP, (f): CPP-ACP +Listerine mouth wash. The testing procedures were used surface micro hardness testing and environmental Scanning electron microscope.

Result: All remineralizing agents groups had a lowest statistically significant surface micro hardness than control groups. The CPP-ACP + Listerine total care zero mouth wash showed a higher statically significant surface micro hardness than the negative control.

Conclusion: Consistent with clinical findings, when applied only to demineralized teeth in this study, The CPP-ACP and CPP-ACPF should to have powerful effect in enhancing surface structure and micro hardness of demineralized enamel surface and External fluoride present in mouth wash has more synergetic effect than the conjugated fluoride in CPP-ACPF in demineralized enamel surface remineralization.

KEYWORDS Environmental Scanning Electron Microscope, Remineralization, Listerine Total Care Zero, CPP-ACP, CPP-ACFP

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INTRODUCTION

Dental caries is a microbial illness of the calcified tissue of the teeth that extends into the sub-surface and is characterized by demineralization of the inorganic portion of the tooth and destruction of the calcium organic substance.^[1]

Salivary dysfunction, cariogenic bacteria, and fermentable carbohydrates are all recognized as key pathogenic contributors. Because of the imbalance, the physiological processes of remineralization and demineralization of the tooth structure would be disrupted, favouring the latter.^[2]

The focus on caries has switched to developing approaches for detecting early-stage caries lesions and using non-invasive therapy for these lesions.

The natural repair process of restoring minerals to the hydroxyapatite's latticework structure in the form of mineral ions is known as remineralization. Fluoride is a remineralizing agent that interacts with oral fluids on the interface of enamel and subsurface areas of teeth and forms fluorapatite crystals by combining calcium and phosphate ions.

Remineralization as a non-invasive treatment for early carious lesions has the potential to be a significant development in the therapeutic management of the disease.^[3] Based on the remineralization potential of fluoride, casein phosphopeptide amorphous calcium phosphate (CPP-ACP), and casein phosphopeptide amorphous calcium phosphate with fluoride, there is some evidence (CPP-ACPF). When used in conjunction with fluoride toothpaste, CPP-ACP remineralized early enamel lesions and showed improved remineralization capacity.

As a result, the effect of various remineralization treatments (CPP-ACP, CPP-ACPF, and fluoride mouthwash) on demineralized enamel surface micro hardness and morphology must be investigated.

AIM OF THE STUDY

This study was conducted to evaluate the effect of different remineralization protocols:

Casein phosphopeptide- amorphous calcium phosphate (CPP-ACP) (*MI Paste*), Casein phosphopeptide- amorphous calcium phosphate with fluoride (CPP-ACFPF) (*MI Paste Plus*), fluoridated mouth wash (Listerine total care zero), Casein phosphopeptide- amorphous calcium phosphate (CPP-ACP) +Fluoridated mouth wash (Listerine total care zero) on demineralized enamel surface micro hardness.

MATERIAL AND METHODS

Materials

TABLE (1): Materials, Description, manufacture

Materials	Description	Manufacture
GC MI past (RECALDENT)	Remineralizing agent: casein phosphopeptide amorphous calcium phosphate CPP-ACP	GC Japan
GC MI past plus (RECALDENT)	Remineralizing agent: casein phosphopeptide amorphous calcium fluoride phosphate CPP-ACFP	GC Japan
Listerine Total care zero mouth wash	Sodium fluoride (220 ppm F)	Italy

Methods:

Preparation of Enamel Specimens

Thirty non-cariou, freshly extracted human premolars for orthodontic purposes were used as samples in this investigation.

The samples were cleaned and stored in distilled water until they were used. Using a diamond disc and copious water spray, the roots of the teeth were decoronated at the level of the cemento-enamel junction. Crowns were cut into two equal parts vertically. Each tooth's buccal and lingual portions were embedded in acrylic resin so that the enamel surface faced upward.^[4] Finally, the buccal side was flattened and polished using 600, 800, and 1000 grit

abrasive papers (latexed waterproof paper brown aluminium oxide electro coated, china) before being divided into six groups of ten samples each.

Specimens Grouping and Study Design

The specimens were randomly divided into the following six treatment groups (n=10):

- **Group A:** Sound enamel (no treatment) (positive Control)
- **Group B:** Specimens were treated with demineralization solution only (negative control)
- **Group C:** Specimens were treated with Listerine Total care zero mouth wash
- **Group D:** Specimens were treated with *GCMIPaste™*, *RECALDENT™*)
- **Group E:** Specimens were treated with *GCMIPaste Plus™*, (*RECALDENT™*)
- **Group F:** Specimens were treated with *GCMIPaste™* + Listerine Total care zero mouth wash

Artificial Demineralization of Enamel Specimens

The demineralizing solution was made by combining the following ingredients: (2.2 mm calcium chloride, 2.2 mm potassium dihydrogen orthophosphate dehydrate to 0.05 M Acetic acid). 1 m potassium hydroxide was used to adjust the pH to 4.4. To create lesions, the specimen was stored individually in 25 mL glass bottles containing 10 mL of demineralization solution for 96 hours.^[5]

Application of Remineralization Different Protocols

The specimens in Groups C, D, E, and F were treated with remineralizing agents every 24 hours for 7 days, as stated in (Table 1). Specimens in D and E were rubbed for 4 minutes with the respective remineralizing agents *CPP-ACP (GC Japan-141208V)* and *CPP-ACFP (GC Japan-151207S)* using a polishing cup attached to a contra-angle hand piece, then cleaned with deionized water for 1 minute before being placed in artificial saliva. Sato et al. [6] produced artificial saliva. The specimens in

C (*Italy-3136P*) were given a one-minute treatment once a day. Specimens in group F were exposed to *CPP-ACP* for 4 minutes and then rinsed in deionized water for 1 minute before receiving a mouth rinse treatment for one minute and being placed in 10 ml of artificial saliva. Only deionized water was used to wash the positive and negative control groups. Artificial saliva was replaced every 24 hours before freshly treated samples were immersed.

TESTING PROCEDURES

Surface Hardness Testing

The micro hardness of two control groups (positive control and negative control) and remineralization groups treated with Listerine total care zero mouth wash, *CPP-ACP*, *CPP-ACFP*, and *CPP-ACP* + Listerine total care zero mouth wash for a period of seven days was assessed using a Digital Vicker Hardness Tester (*Nexus 4000™*, *INNOVA TEST*, model no .4503, Netherlands) Force application (10 s, 100 g load). The Vickers hardness number was determined by allowing the laden diamond to sink and rest on the enamel. **Fig (1)**



Fig. (1) Specimen placed in microhardness testing machine

Environmental Scanning Electron Microscope

The enamel surface was scanned using ESEM magnification (x5000), (*SEM Model Quanta 250 FEG (Field Emission Gun)* attached with *EDX Unit (Energy Dispersive X-ray Analyses)* with an

accelerating voltage of 30 K.V., FEI Company, Oregon, USA) to evaluate surface morphology (negative control, demineralized group treated with remineralization agents).

Statistical Analysis

The experimental (micro hardness) results were statistically analyzed with SPSS (21st edition, IBM Corporation, New York, USA).

The effect of the application period (7 days) of remineralization agent (CPP-ACP, CPP-ACFP, Listerine total care zero, and CPP-ACP + Listerine total care zero) on the micro hardness of enamel was evaluated using a One-Way ANOVA followed by a Tukey HSD post hoc test.

RESULTS

Result of Micro Hardness Test

The effect of the 7-day application period of remineralizing agents (CPP-ACP, CPP-ACFP, Lis-

terine total care zero mouth wash and CPP-ACP +Listerine total care zero mouth wash) on the micro hardness of enamel was evaluated using an On-Way ANOVA followed by a Tukey HSD post hoc test. It was discovered that the degree of mineralization has a statistically significant impact on micro hardness, as shown in **Table (2)**.

Tukey HSD post hoc test revealed that, All remineralizing agents groups had the lowest statistically significant surface micro hardness than control groups (180.21a36.02), while CPP-ACP + Listerine total care zero mouth wash had the highest statistically significant surface micro hardness (121.97b 28.07) than negative control (68.16 c14.57) and Listerine total care zero mouth wash groups (63.60c15.86), but there was no significant difference between CPP-ACP, CPP-ACFP, the CPP-ACP + Listerine total care zero mouth wash groups as shown in **Table 3, Fig(2)**

TABLE (2): The effect of the application (7 days) of remineralizing agents on the micro hardness of demineralized enamel

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	64708.180 ^a	5	12941.636	20.937	.000	.714
Intercept	541440.107	1	541440.107	875.954	.000	.954
mineralization	64708.180	5	12941.636	20.937	.000	.714
Error	25960.806	42	618.114			
Total	632172.529	48				
Corrected Total	90668.986	47				

R Squared = .714 (Adjusted R Squared = .680), sig= significant at p= 0.05, df= degree of freedom, F= force

TABLE (3): Mean ± standard deviation values of the effect of remineralization on the micro hardness of, sound and demineralized enamel groups and remineralizing groups

Groups	Sound enamel (Positive control)	Demineralized Enamel (Negative control)	CPP-ACP	CPP-ACFP	Listerine Total care zero Mw	CPP-ACP +Listerine total care zero Mw
Mean ± Standard deviation	180.21 ^a ±36.02	68.16 ^c ±14.57	103.91 ^b ± 18.41	103.93 ^b ± 30.74	63.60 ^c ±15.86	121.97 ^b ± 28.07

Same lower case superscript letters indicate no statically significant different (P<0.05)

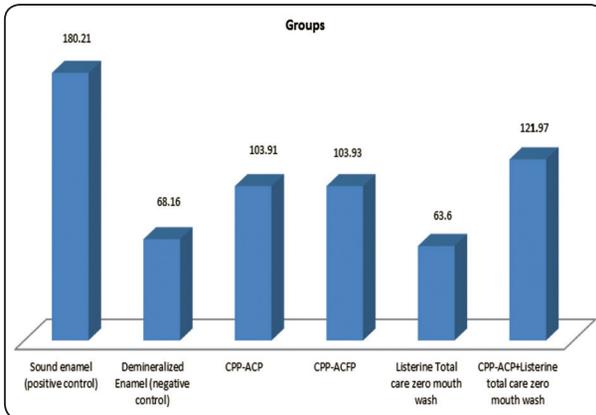


Fig. (2): Bar chart for the effect of the period of application (7 days) of remineralizing agents (CPP-ACP, CPP-ACFP and Listerine total care zero mouth wash and CPP-ACP + Listerine total care zero mouth wash) on the microhardness of demineralized enamel.

Environmental Scanning Electron Microscope

Each experimental group (positive control, negative control, groups treated with CPP-ACP, CPP-ACFP, Listerine total care zero mouth wash, and CPP-ACP + Listerine total care zero mouth wash) had a randomly selected specimen scanned under an ESEM for evaluation of enamel surface. Representative ESEM images are shown in **Fig (3-A-F)**. According to the substance, the degree of the enamel surface morphology differed. Under (5000x) magnification, the typical enamel surface appears smooth (key hole appearance) (**Fig 3A**), however the enamel surface treated with demineralization solution appears irregular, roughened, and has depressed enamel prism cores (**Fig 3B**) while an ESEM photomicrograph of a demineralized enamel surface treated with CPP-ACP revealed parts areas of reformation of the characteristic key hole appearance of the end of the enamel rod (**Fig 3C**) and CPP-ACFP revealed all areas of reformation of the characteristic key hole appearance of the sound enamel surface (**Fig 3D**) and CPP-ACP + Listerine total care zero mouth wash revealed areas of reformation of the characteristic key hole appearance of the sound enamel surface (**Fig 3E**) and Listerine total care zero mouth wash showed deposition of minerals with an unclear and unidentified surface appearance. (**Fig 3F**)

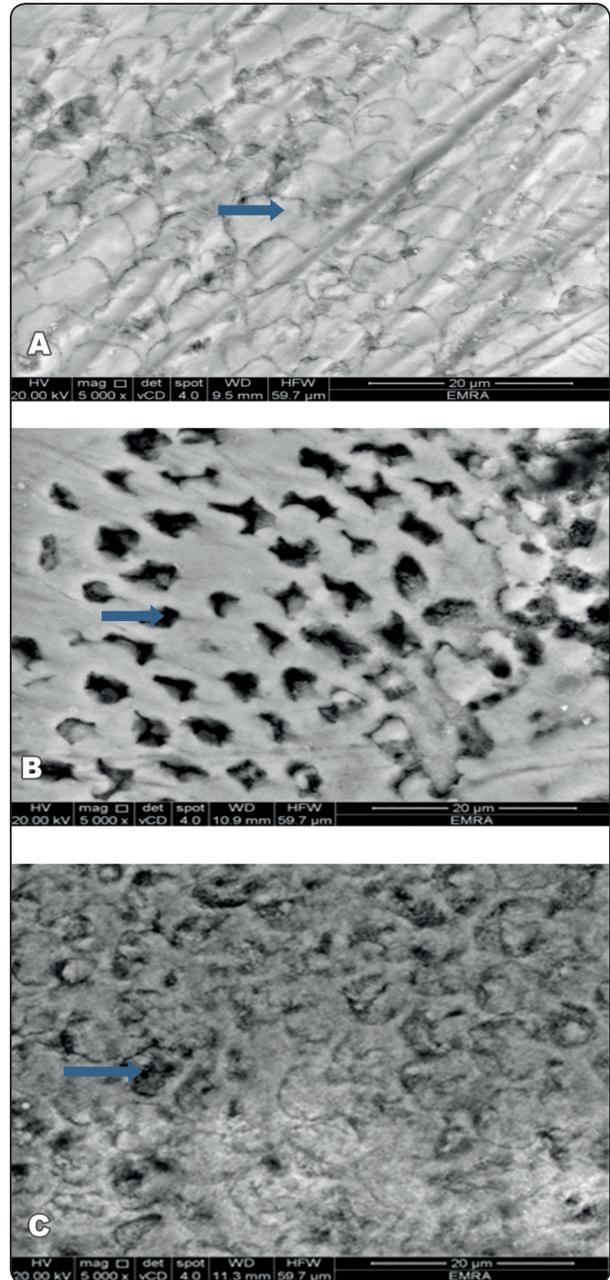


Fig. 3A: ESEM micrographs of the normal enamel under (5000x) magnification showed the enamel surface look smooth (Key hole appearance).

Fig 3B: ESEM microphotographs of the enamel surface treated by demineralization solution under (5000x) magnification showed irregular, roughened appearance with depressed enamel prism cores (blue arrow)

Fig 3C: ESEM photomicrograph of demineralized enamel surface treated with CPP-ACP for 7 days under (5000x) magnification. Parts Areas showing of reformation of characteristic key hole appearance of the end of enamel rod (blue arrow)

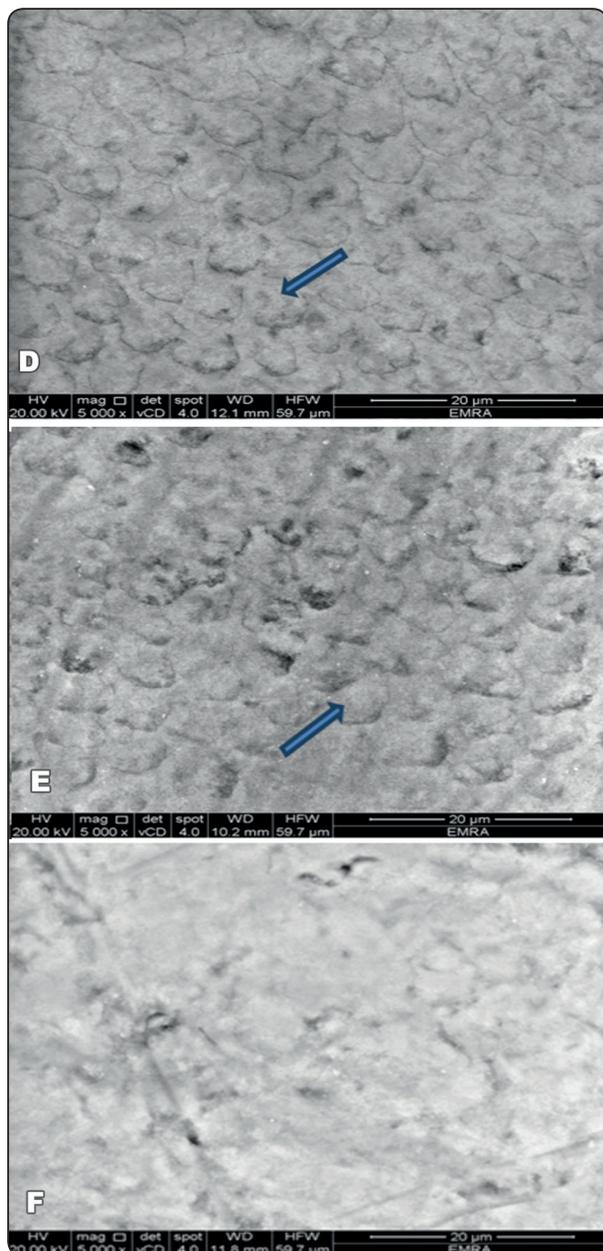


Fig 3D: ESEM photomicrograph of surface treated with CPP-ACFP for 7 days under (5000x) magnification all areas showing reformation of characteristic key hole appearance of the sound enamel surface (Blue arrow).

Fig 3E: ESEM photomicrograph of surface treated with CPP-ACP +Listerine total care zero mouth wash for 7days under (5000x) magnification, it showed areas reformation of characteristically key hole appearance (blue arrow).

Fig 3 F: ESEM photomicrograph of surface treated Listerine total care zero mouth wash under (5000x) magnification showed deposition of minerals with unclear and unidentified surface appearance

DISCUSSION

Dental caries pathophysiology is a dynamic process marked by alternating periods of demineralization and remineralization, rather than a continuous progressive loss of tooth minerals.

In the presence of calcium and phosphate ions, fluoride ions can help remineralize the lost mineral in early caries lesions. However fluoride alone cannot remineralize.^[7]

LISTERINE® has been on the market for decades, with the original version accessible throughout the 1900s, so there is a considerable body of evidence to support its usage. Much clinical research has been done on LISTERINE®.^[8]

For in vitro and in situ dental research, specimens made from human teeth (premolar teeth) for orthodontic purposes are chosen because they allow for testing of the study hypothesis in a more clinically appropriate substrate and simulation of the oral environment.

To mimic the oral environment, artificial saliva was chosen as a storage medium. Sato et al. produced a composition that was used in this study.^[6]

Micro hardness studies have been frequently utilized to assess changes in enamel following tooth-paste treatment.^[9] Micro hardness testing of enamel and dentin does not have a standard condition. The micro hardness of hard tooth tissues has been measured using indentation micro hardness testing using either a Vickers or a Knoop indenter. However, it has been suggested that the Vickers test is more relevant in studies of enamel micro hardness.^[10]

There are two ways for detecting demineralization: (1) invasive and (2) noninvasive.^[11] White spot lesions aren't visible until they've developed 200-300 μm through the enamel.

The image analysis technique (SEM) allows for accurate measurements and can generate detailed and objective data by evaluating a large number of variables.^[12]

In this study the specimens were immersed in the demineralizing solution for 96 hours. This resulted in subsurface demineralization and a drop in the mean Vickers hardness numbers of the enamel surface, which is consistent with the theory of decreased enamel hardness as a result of mineral loss caused by the demineralization process.^[13]

SEM morphologic examination revealed (**Fig.3B**) that enamel subjected to demineralization solution exhibited an early pattern of demineralization with indications of interprismatic mineral loss^[14]. The amount and duration of acidic attacks, as well as the acidic agent's pH value, all influence tooth substrate erosion.^[15]

In this study, fluoride, contrary to popular belief, had no effect on remineralization; nonetheless, CPP-ACP + Listerin total care zero mouth wash was one of the best results, with little difference between CPP-ACP and CPP-ACP with fluoride. CPP-ACP + fluoride and CPP-ACP alone have no effect in comparison to each other.

Our findings are in line with those of Rirattanpong et al [16], who found no difference between utilizing CPP-ACP with fluoride ions and using CPP-ACP alone. Srinivasan et al,^[17] Lussi^[18], and Kumar et al,^[19] on the other hand, These findings imply that CPP-ACP in conjunction with fluoride has a stronger remineralizing capacity than CPP-ACP alone.

In our study, when CPP-ACP was administered as a topical coating followed by treating the lesions with the fluoride mouth wash, the increase in remineralization was higher in the CPP-ACP+Listerine total care zero mouth wash group (121.97 28.07) than in the Listerine total care zero mouth wash group. This was most likely owing to CPP-ACP capacity to interact with fluoride ions, resulting in the creation of a stable amorphous calcium fluoride phosphate phase, which had an added anticariogenic impact. According to some research, when fluoride was added to the CCP-ACP, the acid-resisting action was improved. The micro

hardness of the Listerine comprehensive care zero mouth wash group was also found to be the lowest compared to the other groups. Low pH dental care products, according to Zero^[20], may be erosive if used repeatedly.

In the present study, after treatment with paste containing CPP-ACP or CPP-ACFP, favourable changes in surface micro hardness were seen in comparison to negative groups. When compared to demineralized enamel, Rehder Neto et al^[21] found that the tested compounds had a substantial effect, with calcium sodium phosphosilicate being the most effective.

After treatment with paste containing either CPP-ACP or CPP-ACFP and (CPP-ACP + Listerine total care zero mouth wash), SEM observations confirmed alterations in enamel surface morphology, with a reduction in enamel defect size and plugging of porous defects, resulting in a decrease in cavities and micropores and reestablishment of surface integrity. In comparison to the demineralization group, distinct surface coatings deposited by different agents were visible and did not indicate enamel disintegration. These findings are congruent with observations that CPP-ACP and CPP-ACFP enable the development of partially demineralized hydroxyapatite crystals, indicating enamel remineralization. The enamel treated with CPP-ACFP has a homogeneous smooth appearance when compared to CPP-ACP. Enamel rods and prismatic substances are not discernible in the CPP- ACPF group, according to Jayarajan et al.,^[22] but patches of calcified deposits are more visible and concentrated along the porosity flaws.

CONCLUSION

Within the limitations of this study, it was concluded that:

- 1- fluoride mouthwash was insufficient in improving the surface structure and microhardness of artificially demineralized enamel.

2- CPP-ACP and CPP-ACPF have a significant impact on the surface structure and microhardness of artificially demineralized enamel. Finally, in artificially demineralized enamel surface remineralization, external fluoride in mouthwash had a greater synergetic effect than conjugated fluoride in CPP-ACPF.

RECOMMENDATIONS

Further clinical studies are required to improve the remineralization ability of CPP-ACP and CPP-ACPF to be comparable to the various commercially available remineralizing agents.

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