# Comparative *In Vitro* Evaluation of The Novel Remineralizing Agents' Effects on Enamel Surface Hardness

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**Abstract**

Remineralizing agents have been recommended as preventive and therapeutic agents in the management of demineralization, but their efficacy to increase the surface hardness of enamel surface has not been evaluated. The current study was designed to evaluate the effect of three new age remineralizing agents such as fluoride enriched Casein Phosphopeptide – Amorphous Calcium Phosphate (GC Tooth mouse Plus) and beta-tri calcium phosphate (ClinPro) and fluoride rich hydroxyapatite (ReminPro) based remineralizing agents on enamel surface hardness. Sixty Tooth sections measuring 4x4x2 mm to harvest enamel were prepared on Intact caries free human premolars extracted for orthodontic purposes from 17 patients in the age group of 15-30 years. Except the sections in control group, all the other sections were subjected to demineralization followed by remineralisation by CPP-ACPF, beta-tri calcium phosphate and fluoride rich hydroxyapatite based remineralizing agents. Enamel surface hardness was calculated using Vickers micro hardness testing.

Among three subgroups, CPP-ACP (331.5 VHN) has increased enamel remineralization potential when compared to both ClinPro (281.5 VHN) and ReminPro (313.6 VHN). In multiple comparison between subgroups p value was highly significant (<0.0001).

CPP-ACPF has increased enamel remineralization potential when compared to both ClinPro and ReminPro and ReminPro is a better remineralizing agent on comparison with ClinPro.

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## Introduction

In the oral environment, tooth structure continuously undergoes intermittent phases of demineralization and remineralization.[1] If the balance is disrupted, demineralization will progress leading to deterioration of the tooth structure.[1] Both dental caries and erosion are initiated by a process called as demineralization which results from the dissolution of mineral phase of tooth structure.

Remineralization is the natural repair process for non - cavitated lesions and relies on adding calcium and phosphate ions assisted by fluoride to rebuild a new surface on existing crystal remnants in subsurface lesions remaining after demineralisation.[2,3,4] These remineralized crystals constituting chiefly of calcium/phosphate elements are less acid soluble than the original mineral and contribute to increased surface hardness depending on the extent of mineralization. Traditionally fluoride has been effectively used as a protective and anti-caries agent with great success. Enamel subsurface lesions need to be diagnosed as early as possible for the best possible remineralization of enamel and to restore good functioning of the tooth.[5] Now, there is much evidence which indicates that the caries preventive action of fluoride is primarily post eruptive through the topical effect, which includes inhibition of demineralization, enhancement of remineralization and inhibition of bacterial activity in the plaque.[6]

Research indicates that topicals which are used frequently, such as daily use of dentifrices and mouth rinses can enhance remineralization

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and retard demineralization of enamel.[7-8] The marked caries reduction in many countries over the last three decades is thought to be mainly the result of the widespread and frequent use of low concentrations of fluorides mainly via toothpastes.[9-10] In the past few years, several new remineralizing agents have been introduced based on unique and varying formulations such as Casein milk protein, tri calcium phosphate and hydroxyapatite– based complex. Several studies have been conducted on the use of these remineralizing agents[11-12] to replace the lost minerals in the management of demineralization of enamel arising from dental erosion, dental caries etc[10] and have shown their superior benefits compared to the conventional fluoride – based agents.[7] According to the study conducted by Brian A. Burt posteruptive action was soon evident due to consistent data from laboratory to epidemiology. The posteruptive hypothesis can be readily adopted as the primary mechanism for fluoride's anticariogenic action.[13]

With the advent of the various formulations of remineralizing agents, it is not only important to evaluate their remineralizing abilities but also their effect on the properties of enamel such as surface hardness as it is constantly exposed to mechanical forces like abrasion and attrition. To study various changes taking place in enamel during demineralization and remineralization, it is necessary to carry out at least part of the experiments under in –vitro conditions. Hence, this in-vitro study has been designed to evaluate and compare the effect of three different novel remineralizing agents such as fluoride enriched Casein Phosphopeptide – Amorphous Calcium Phosphate (CPP-ACPF),[5] beta-tri calcium phosphate and fluoride rich-hydroxyapatite based remineralizing agents on enamel surface hardness.

## Materials and methods

### Selection of sample

Before the start of the study, Ethical Clearance was obtained from Institutional Ethical Board. Freshly extracted human maxillary premolar teeth extracted from 17 individuals in the age group of 15-30 years on orthodontic grounds were collected from the Department of Oral & Maxillofacial Surgery of the institute. Teeth with dental caries, non –carious lesions such as abrasion, attrition and erosion, hypoplasia, restorations, developmental defects, discolorations and traumatic injury/ cracks were excluded from the study.

The teeth were subjected to heat sterilization for a period of 20 days in accordance with the infection control protocol based on OSHA (Occupational Safety and Health Administration ) guidelines.[15] Teeth were stored in Isotonic saline (0.9% Sodium Chloride / NaCl) till the start of the study.

### Sample Preparation

Enamel samples were prepared by slicing the buccal surfaces of the selected teeth, 60 teeth were used and 60 samples were obtained from each tooth. using a double faced diamond disc mounted on a contra-angle hand piece. Following sample preparation windows were created on the buccal surfaces (dimension of 4x4 mm) using adhesive tape and the sample was made completely and all the other surfaces were made resistant to acid attack by impermeable coating material (Colorama nail varnish, Maybelline). After drying the adhesive tape was removed from the enamel using a sharp tipped instrument and the rectangular area exhibiting the enamel surface was exposed and cleaned using Isotonic saline (0.9% Sodium Chloride) The enamel samples were randomly divided into five groups of 10 samples each (n=50). And the groups were divided as:

Group IA -No surface treatment (control): n=10
Group IB - Samples will be subjected to demineralization: n=10
Group II A -Surface treatment with CPP-ACPF (GC Tooth Mousse Plus): n=10
Group II B -Surface treatment with Beta-TCP (ClinPro): n=10
Group II C -Surface treatment with Hydroxyapatite (ReminPro): n=10

### Demineralization and Remineralization process:

Each of the enamel samples from group IB, IIA, IIB and IIC were then immersed in 40 ml of demineralizing solution [Acetate 0.1Mol/L, Calcium 0.1Mol/L, Phosphate 0.1Mol/L, Fluoride 0.1mg/L, pH 5.5] for a period of continuous 4 days at a constant temperature of 37 degree centigrade, in an incubator to induce artificial

caries formation, simulating an active area of demineralization**.** Each of the samples were washed with de-ionized water for a minute and dried with an air-jet for 10 seconds. Enamel samples from the group IIA, IIB and IIC were treated with the respective remineralizing agents [CPP-ACPF (GC Tooth Mousse Plus), Beta-TCP (ClinPro), Hydroxyapatite (ReminPro)] on the prepared windows on the buccal surfaces. Remineralization process was done in conjugation with pH cycling following which all these samples were subjected to

**pH Cycling**:

pH cycling was done to mimic the natural process of cycles of demineralization and remineralization that occurs in the oral environment in the presence of saliva. The samples were first immersed in 20ml of demineralizing solution (Calcium 2.0mMol/L, Phosphate 2.0mMol/L, Acetic acid 75.0mMol/L, pH 5.5) for a period of 3 hours followed by immersion in a remineralizing solution a period of 4 minutes (1.5mM Calcium, 0.9mM Phosphate, 0.15 M KCl in 0.1M Tris buffer, pH 7) for around 30ml per sample. During the intermittent intervals the samples were stored in Artificial Saliva (pH 7.0). This completes one pH cycle. This cycle was repeated twice daily for a period of 14 days.
The remineralizing solution was replaced every 48 hours and the demineralizing agent replaced every 5 days. Samples from Group IA and IB were not surface treated with remneralizing agents were stored in artificial saliva for 14 days.
Hardness Testing:

On the 15th day all the samples (Group IA, IB, IIA, IIB and IIC) were subjected to surface micro hardness testing. Evaluation of surface micro hardness was done on the on the buccal surface of the tooth surfaces using Vickers hardness test (Micro Vickers Hardness Tester, Matsuzawa. Co. Ltd).
Flat surface of enamel specimen was impressed with loads of 200g for 10seconds

**Statistical Analysis**

The data was collected and transferred from pre-coded proforma to computer. The data was analyzed using SPSS (IBM) version 23. Descriptive statistics included Mean, standard Deviation. Inferential statistics included independent One Way ANOVA for multiple analysis with Bonferroni corrections. The level of significance was set at 0.05 at 95% confidence intervals.

**Results**

Table 1 describes the mean values of all the groups where highest surface hardness was found in intact enamel that is 340.8 $kg.mm^{-2}$ VHN. Demineralized enamel sample showed a surface hardness of 230.5 $kg.mm^{-2}$ which is the least. Among the samples which were treated with remineralizing agents, CPP-ACPF showed the highest mean value of 331.5 $kg.mm^{-2}$ VHN followed by Hydroxyapatite and Beta-TCP with mean VHN values of 313.6 $kg.mm^{-2}$ and 281.5 $kg.mm^{-2}$ respectively. Multiple group comparisons were done using one way ANOVA which showed a highly significant difference ($p<0.001$) between the groups.

In the table 2, Based on the mean values, intact enamel and demineralized enamel samples showed a significant difference ($p<0.001$). There was also a significant difference ($p<0.001$) between the mean values of intact enamel and beta –TCP. In comparison with the mean values of demineralized enamel with that of CPP-ACPF and Hydroxyapatite, both showed a significant difference ($p<0.001$). Also there was a significant difference between the mean values of CPP-ACPF and Beta-TCP ($p=0.003$).

| Group | N | Mean | Standard deviation | F value | p value |
|---|---|---|---|---|---|
| Intact Enamel | 10 | 340.8 $kg.mm^{-2}$. | 19.71 | | |
| Demineralized Enamel | 10 | 230.5 $kg.mm^{-2}$. | 15.87 | 24.842 | P <0.001 (HS) |
| CPP-ACPF | 10 | 331.5 $kg.mm^{-2}$. | 35.90 | | |
| Beta-TCP | 10 | 281.5 $kg.mm^{-2}$. | 39.94 | | |
| Hydroxyapatite | 10 | 313.6 $kg.mm^{-2}$. | 22.72 | | |

**Table 1.** Descriptive measures such as Mean values of surface hardness and One way ANOVA for multiple comparisons.

**Discussion**

In the present investigation, control group (Intact Enamel) showed the highest surface hardness while Demineralized Enamel Group, which was pre-treated with a demineralizing solution of pH 5.5 showed the lowest surface hardness (**230.5**$kg.mm^{-2}$). This demonstrates that the tooth tissue subjected to demineralization

undergoes the significant loss of surface hardness when compared to that of intact tooth structure. The results are in agreement with the study done by Rolla G.B. et al,[6] which showed that surface hardness decreased under different acidic conditions. Another study by PojjanutBenjakul et al,[16] showed the association of enamel loss with the pH and TA of beverages and both parameters can be used to predict the qualitative erosive potential of different beverages.

CPP-ACPF showed the significantly higher value of surface hardness when compared to the group surface treated with ClinPro. These results are in agreement with the study conducted by Reinsema and Arends et al., which showed higher surface hardness with the surface treated with CPP-ACP than that of with ClinPro.[17] It is also in agreement with study conducted by Sule Bayrak et.al[18] reported that ClinPro(beta-TCP) fluoride varnishes were all found to have positive effects on the prevention of enamel erosion, however, the fluoride varnish containing CPP-ACP was the most effective in increasing the enamel's resistance to erosion.

All the remineralizing agents tested are fluoride containing newer formulations. The CPP-ACPF has been shown to remineralize enamel subsurface lesions in situ when delivered in oral care products. The level of fluoride is 900ppm which approximates that of adult tooth pastes. CPP-ACP binds to bio films, plaque, hydroxyapatite localizing bioavailable calcium, fluoride and phosphate. Tri calcium phosphate has been considered as one possible means for enhancing levels of calcium and phosphate in saliva. Functionalized Tri calcium phosphate comprises of beta TCP and sodium lauryl sulphate (SLS) with fluoride level of 950 ppm.[19,20]

ClinPro showed the lesser value of surface hardness when compared to the group surface treated with ReminPro. Beta- TCP is a recently introduced remineralizing agent which is a precursor to hydroxyapatite formation. It is biocompatible, bioactive and manifests lattice defects that allow for crystal modification.[21,22] It is a new hybrid material with milling technique that fuse beta TCP and sodium lauryl sulphate. According to manufacturer when TCP comes in contact with the tooth surface, moistened by saliva protective barrier breaks down, making the calcium phosphate and fluoride ions available to the teeth for remineralization of tooth surface.[23] Amongst the two groups, surface treated with ReminPro showed higher value of surface hardness when compared to the group surface treated with ClinPro. This is in agreement with the study by Rahul Rao et al,[24] ClinPro, Duraphat, and ReminPro were 54.88%, 43.42%, and 26.86%, respectively. The difference in the percentage for ClinPro paste was better than Duraphat and ReminPro, and this difference was statistically significant ($p<0.05$). ClinPro tooth Crème showed the best remineralization potential among the three materials tested followed by Duraphat and ReminPro.

HA crystals exhibit high levels of biomimetic properties due to their composition, structure, morphology, bulk and surface physical–chemical properties. HA are bioinspired molecules that has a surface area of 100 $m^2/g$, which makes them possess strong affinity to the demineralized surfaces.[25] The SEM analysis of the surface after remineralization induced by nanohydroxyapatite reflects this observation where HA nanocrystals were found to adhere to the pores created by demineralization[4]. These adherent nanocrystals were found to aggregate and grow into microclusters and form a uniform apatite layer on the demineralized surface. The surface also revealed the newly formed apatite layer to be completely covering the prismatic and interprismatic enamel structures.[25] The same observation was noticed in an earlier study where 100 nm carbonated HA particles were found to cover the demineralized enamel surfaces more effectively when compared with fluoridated toothpaste.[26]

In the present study, the demineralising solution used was regulated to a pH value of 5.5, which is referred to as critical pH.[27] This critical pH is a pH at which a solution is just saturated with respect to particular mineral, such as tooth enamel. Plaque is normally supersaturated with respect to tooth enamel because the pH is lower than the critical pH. In-Vivo conditions, dental plaque contains higher concentrations of calcium and phosphate than that of saliva and its critical pH may be as low as 5.1 and in the present study, teeth are not dissolved in plaque. Hence the results obtained in the current study may be varied if the pH is changed. Hence further investigation may be warranted to evaluate the results under variable pH values.

Several in vitro studies have shown the remineralizatioon of artificially demineralised

enamel lesions as in the study conducted by Feagin et al.[28] In-Vivo remineralization also has been observed in the study conducted by Koulourides et al.[29] Our results are in agreement with these experimental results from these earlier studies in which the experimental conditions for remineralization were favourable when compared with caries lesions on smooth surface.

To study various changes taking place on the tooth surface during demineralization and remineralization, it is necessary to carry out at least part of experiment under in vitro conditions using advanced techniques such as micro-radiography, micro-tomography, detection of chemical changes using Atomic absorption spectrometer (AAS), Scanning electron microscopy (SEM), Energy Dispersive X-ray Analysis (EDAX). Although evaluation of remineralization can be done by several of these methods, in the present study we have used a simple method of evaluating surface micro hardness as preliminary evaluation of comparative remineralization.

The advantage of an in vitro model is that it gives well controlled experimental conditions; however, it fails to amount for the complexity of in vivo conditions. Since the demineralization in this experiment was induced under in vitro conditions, the results of this study cannot be directly compared to an in vivo situation. Hence further studies that simulate in vivo conditions more closely followed by long term clinical trials are recommended for conclusive results.

## Conclusions

Within the limitations of this study, we conclude that: Highest Enamel surface hardness was shown by CPP-ACP agents and the mean value was close to Intact Enamel (control), CPP-ACP has increased enamel remineralization potential by increasing enamel surface hardness compared to both ClinPro and ReminPro. Among Clin Pro and Remin Pro, ReminPro is a better remineralizing agent on comparison with ClinPro. A goal of modern dentistry is the non‐invasive management of non‐cavitated caries lesions involving remineralization systems to repair the enamel with fluorapatite or fluorhydroxyapatite. With a clearer understanding of the implementation of these remineralizing agents, we can create a more favorable relationship in which remineralization can occur. It is important for dental professionals to be aware that it takes significant time to establish the bonafides of a new technology.

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## Declaration of Interest

No Conflict of Interest.

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| | | Mean difference | Standard error | Sig. | 95% Confidence interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound |
| Intact Enamel | Demineralized Enamel | 110.55000 | 12.71461 | .000 | 73.0155 | 148.0845 |
| | CPP-ACPF | 9.55000 | 12.71461 | 1.000 | -27.9845 | 47.0845 |
| | Beta-TCP | 59.30000 | 12.71461 | .000 | 21.7655 | 96.8345 |
| | Hydroxyapatite | 27.20000 | 12.71461 | .379 | -10.3345 | 64.7345 |
| Demineralized Enamel | CPP-ACPF | -101.00000 | 12.71461 | .000 | -137.1279 | -64.8721 |
| | Beta-TCP | -51.25000 | 12.71461 | .002 | -87.3779 | -15.1221 |
| | Hydroxyapatite | -83.35000 | 12.71461 | .000 | -119.4779 | -47.2221 |
| CPP-ACPF | Beta-TCP | 49.75000 | 12.71461 | .003 | 13.6221 | 85.8779 |
| | Hydroxyapatite | 17.65000 | 12.71461 | .638 | -18.4779 | 53.7779 |
| Beta-TCP | Hydroxyapatite | -32.10000 | 12.71461 | .152 | -69.6345 | 5.4345 |

**Table 2.** Post Hoc test with Bonferroni corrections for multiple groups comparisons.

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