

# Comparison of Remineralization Potential of Casein Phosphopeptide: Amorphous Calcium Phosphate, Nano-hydroxyapatite and Calcium Sucrose Phosphate on Artificial Enamel Lesions: An *In Vitro* Study

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## ABSTRACT

**Aim and objective:** To evaluate and compare the remineralization potential of CPP-ACP, nano-hydroxyapatite, and calcium sucrose phosphate toothpaste on artificial enamel caries lesions by means of microhardness testing.

**Materials and methods:** Twenty sound human primary molars, extracted for therapeutic reasons were selected for this study. From each tooth, two enamel specimens were prepared and embedded in acrylic resin blocks, and each block contains five tooth samples. After polishing, the baseline hardness of the enamel surface (KHN) was determined by Knoop microhardness testing. Then the specimens were randomly assigned into four groups ( $n = 10$ ), according to the remineralizing agent used: **group I:** Control, **group II:** GC Tooth Mousse<sup>TM</sup> (CCP-ACP), **group III:** Acclaim<sup>TM</sup> (nano-HAP), and **group IV:** EnaFix (CaSP). The specimens were then immersed in a demineralizing solution and post-lesion KHN values were obtained as baseline measurements. Later remineralizing agent was applied and after 7 days of remineralization, posttreatment KHN tests were conducted.

**Results:** Data were analyzed using paired *t*-test, analysis of variance, and Tukey HSD test. Mean enamel surface microhardness (KHN) values after remineralization shows that group IV (EnaFix<sup>TM</sup>) had maximum hardness number ( $114.71 \pm 12.27$ ) followed by group III (Acclaim) ( $85.14 \pm 22.82$ ) and group II (GC Tooth Mousse) ( $56.42 \pm 19.90$ ). The difference was statistically significant ( $p < 0.001$ ). Similarly the %SMHR was also highest in group IV (EnaFix), followed by group III (Acclaim) and group II (GC Tooth Mousse).

**Conclusion:** EnaFix (calcium sucrose phosphate) shows a maximum increase in the enamel surface microhardness followed by Acclaim (nano-HAP) and GC Tooth Mousse.

**Clinical significance:** Calcium Sucrose Phosphate in toothpaste strengthened the enamel more than nano hydroxyapatite and CPP-ACP, and can be an alternative to the use of fluoride toothpaste in children.

**Keywords:** Casein phospho peptide-amorphous calcium phosphate (CPP-ACP), Casein sucrose phosphate (CSP), Microhardness, n-HAP.

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## INTRODUCTION

Dental caries, being the most infectious disease to exist among the decades,<sup>1</sup> its prevalence in a modern society whose chief diet is carbohydrates and other sugary starch is increasing. The process of caries formation is mainly affected by: (1) The interaction between saliva and plaque, (2) A disturbance in the balance of healthy microbial flora, and (3) A change in physicochemical factor of the mineral content of tooth that makes dental hydroxyapatite more prone to caries<sup>1,2</sup>

The ratio between demineralization and remineralization plays important role in maintaining the strength of the tooth. The application of remineralizing agents like casein is the most modern biological approach for the prevention of caries by balancing the demineralization and remineralization ratio.<sup>3</sup>

It is widely accepted that fluoride is one of the most important agents for promoting remineralization, especially with the aid of topical fluorides. Many studies have shown that initial enamel caries lesions can be remineralized by topical application of fluoride agents, including dentifrices, mouthwash solutions, gels, and varnishes.<sup>4,5</sup> Although fluoride presents no problems when used, to a larger extent may lead to fluorosis and further weakening of tooth hence its use should be controlled.<sup>6</sup> So,

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nonfluoridated remineralizing agents were introduced into the market to surpass the disadvantages and limitations of fluoridated agents.

Considering the plethora of nonfluoride remineralizing agents available in the market, this study was conducted for the comparison of remineralization potential of casein phosphopeptide-

amorphous calcium phosphate (CPP-ACP), nano-hydroxy apatite (Nano-HAP), and calcium sucrose phosphate (CaSP) toothpaste on artificial enamel caries lesions by means of microhardness testing.

## MATERIALS AND METHODS

The remineralization potential of three commercially available toothpaste on enamel was analyzed by testing the microhardness with a Knoop microhardness testing machine in an experimental *in vitro* study. The enamel specimens were labeled randomly as one control group and three experimental groups, comprising of 10 teeth each.

- Group I: control group- without a remineralizing agent
- Group II: casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) e.g. GC Tooth Mousse™ (GC Corporation, Tokyo, Japan).
- Group III: nano-hydroxy apatite paste (nano-HAP) e.g. acclaim™ (Group Pharmaceuticals, Karnataka, India).
- Group IV: calcium sucrose phosphate paste e.g. enafix™ (CaSP) (Group Pharmaceuticals, Karnataka, India).

## SPECIMEN PREPARATION AND SELECTION

Following approval from the Institutional Research Ethics Committee, 20 sound human primary molars, which were extracted for therapeutic reasons were selected for this study. Two specimens were taken from the buccal and lingual surface from defectless enamel part of each tooth by using a water-cooled low-speed diamond saw. These specimens were then embedded into acrylic resin blocks and are numbered from 1–8 and each block contains five tooth samples. These enamel surfaces embedded in acrylic blocks were finely ground with water-cooled silicon carbide paper discs (400, 600, and 1200 grit), and 1 µm alumina suspension was used in final polishing. After leaving a window of (3 mm) of the exposed enamel in the center, the rest was embedded in an acid-resistant varnish.

After polishing, the baseline hardness of the enamel surface (KHN) was determined by Knoop microhardness testing with three indentations on the specimens using 25 g-loads applied for 13 seconds. Samples were assigned randomly into four groups ( $n = 10$ ), depending on to the remineralizing agent used:

- Group I: Control group-without remineralizing agent.
- Group II: GC Tooth mousse™ cream (GC Corporation, Tokyo, Japan).
- Group III: Acclaim™ (Group Pharmaceuticals, Karnataka, India).
- Group IV: EnaFix™ (Group Pharmaceuticals, Karnataka, India).

## LESION FORMATION

Initial caries development was induced to obtain incipient lesions before the application of remineralizing agents. The pH-cycling protocol suggested by Vieira AEM et al.<sup>7</sup> with modification, was used in this study. The specimens were kept in a demineralizing

solution [2.2 mM monosodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$ ), 2.2 mM calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 0.05 M lactic acid, and 50% sodium hydroxide to adjust the pH to 4.5] for 6 hours and in artificial saliva (Remineralizing solution) for 18 hours (17.75 mL per specimen) for 2 days. Later deionized water was used for washing after 48 hours and samples were placed in four different glass containers until further evaluation. For obtaining the baseline measurements post-lesion KHN test was done with the same static load and time. Three KHN measurements were performed at various points.

## APPLICATION OF REMINERALIZING AGENT

An amount of 100 mg toothpaste was mixed with 100 mL of distilled water to make remineralization toothpaste slurries. Prior to the cariogenic challenge, to standardize the number of agents applied in each sample different 1 mL insulin syringes were used. In group I, samples was rinsed with deionized water and stored in separate containers with artificial saliva. For group II, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste was brushed with a microapplicator with friction for 10 seconds. After this, CPP-ACP paste was kept in contact with enamel for 5 minutes, followed by rinsing in deionized water then stored in artificial saliva individually. For group III, nano-hydroxyapatite (nano-HAP) paste was applied with a microapplicator with friction for 10 seconds. After this, paste was kept in contact with enamel for 5 minutes, followed by washing with deionized water and stored individually in artificial saliva. For group IV, calcium sucrose phosphate (CaSP) paste was brushed with a microapplicator with friction for 10 seconds and this, the paste was kept in contact with enamel for 5 minutes followed by washing with deionized water and stored individually in artificial saliva. Except for the control group all samples were treated with respective remineralizing agents. At every 24 hours, the artificial saliva was changed prior to immersion of freshly treated sample for the inhibition of fungal growth. The samples were washed with deionized water after 7 days of remineralization and posttreatment Knoop Hardness (KHN) tests were conducted with the same static load and time used to obtain baseline and post-lesion measurements.

## STATISTICAL ANALYSIS

Statistical analysis was tabulated using Statistical Package for Social Sciences (SPSS Version 23.0, Chicago, USA) at 0.05 level of significance. %SMHR (The percentage of Surface Microhardness Recovery) was calculated by the formula:  $100 \times \frac{(\text{Posttreatment Post-lesion})}{(\text{Baseline- Post-lesion})}$ .

The statistical tests used in this study were Paired *t*-test to compare the mean difference between the different time intervals within the group; an analysis of variance (ANOVA) to determine whether there are any statistical differences among the groups and Tukey HSD test to perform pairwise comparisons.

**Table 1:** Baseline, post-lesion, and posttreatment enamel surface microhardness (KHN) in different groups

Groups	<i>n</i>	Baseline	Post-lesion	Posttreatment
Group I (Control)	10	382.14 ± 15.69	13.22 ± 3.69	19.57 ± 10.14
Group II (GC Tooth Mousse)	10	374.07 ± 34.86	16.51 ± 3.73	56.42 ± 19.90
Group III (Acclaim)	10	396.21 ± 33.52	32.29 ± 20.95	85.14 ± 22.82
Group IV (EnaFix)	10	390.06 ± 30.27	18.43 ± 6.25	114.71 ± 12.27

## RESULTS

Table 1 summarizes the mean baseline, post-lesion, and posttreatment enamel surface microhardness values in different groups. It was noticed that the baseline enamel microhardness values ranged from  $374.07 \pm 34.86$  to  $396.21 \pm 33.52$  KHN. On subjecting the enamel specimens to an acid challenge the microhardness values reduced to a range of  $13.22 \pm 3.69$  to  $32.29 \pm 20.95$  KHN after application of the remineralizing agent, the mean enamel microhardness increased in all the groups, with the lowest in the control group and the highest increase was noted in group IV (EnaFix).

The mean difference in the enamel surface microhardness (KHN), at different time intervals for different groups, post-remineralization is summarized in Table 2. After the application of the remineralizing agent, the mean enamel microhardness increased in all the groups with the maximum increase of  $96.28 \pm 14.40$  KHN in group IV (EnaFix). Group III (Acclaim) had a higher post-remineralization microhardness when compared to group II (GC Tooth Mousse). Similarly, the % surface microhardness recovery was also highest in group IV (25.90 KHN), in comparison with other groups (Table 2). When the mean enamel surface microhardness values were compared between the different groups after remineralization, it was noticed that ( $p < 0.001$ ) was highly significant (Table 3). On Tukey test analysis a statistically significant difference ( $p < 0.001$ ) was observed between group I (control) with that of other groups (Table 4). Similarly, there statistically significant difference noticed between group II in relation to group III and IV. there was a statistically significant difference between groups III and IV were also noticed.

## DISCUSSION

The most commonly used method for caries prevention among dentists is the use of fluoride. However, there are concerns about toxicity and adverse effects due to the exposure of fluoride, suggesting that fluoride exposure should be limited.<sup>6</sup> Various nonfluoride remineralizing agents were introduced in the last three decades to have an anticaries protective effect by limiting the demineralizing process and enhancing remineralizing process. Most commonly available nonfluoride pastes in the market are GC Tooth Mousse™ (CPP-ACP), Acclaim™ (nano-HAP), and EnaFix™ (Calcium Sucrose Phosphate) for remineralization of the enamel caries lesions and prevention of dental caries.

In the present study, the mean enamel surface microhardness of primary molars between the group at baseline range from  $374.07$ – $396.31$  (KHN), which clearly shows no significant difference. The mean baseline values were similar to the studies conducted by various investigators<sup>8,9</sup> in primary and permanent teeth using Knoop hardness tester. After demineralization, the enamel surface microhardness values in different groups decreased significantly to a range of  $13.22$ – $32.29$  KHN. The results of our study were similar to the other studies<sup>8-11</sup> reported in the literature. However, in our study, there was a sharp decrease in primary molar enamel surface microhardness compared to the other studies. Since the enamel is less mineralized in primary molars when compared to the permanent teeth there would be a higher rate of dissolution of minerals from the primary molars.<sup>12</sup>

After remineralization with various agents, it was observed that the mean enamel surface hardness increased in all the groups.

**Table 2:** Mean difference in the enamel surface microhardness (KHN) at different time intervals for different groups

Group	Post-lesion-base line	Remineralization-post-lesion	%SMHR
Group I (Control)	$-368.92 \pm 16.64$	$6.34 \pm 6.34$	$\pm 1.72$
Group II (GC Tooth Mousse)	$-357.56 \pm 35.04$	$39.91 \pm 22.76$	$\pm 11.16$
Group III (Acclaim)	$-363.92 \pm 32.51$	$52.85 \pm 18.53$	$\pm 14.52$
Group IV (EnaFix)	$-371.63 \pm 26.92$	$96.28 \pm 14.40$	$\pm 25.90$

**Table 3:** Comparison of enamel surface microhardness between groups after remineralization (analysis of variance)

Source	Sum of squares	df	Mean squares	F ratio	Probability p-value
Between groups	49,521.129	3	16,507.043	56.389	0.000
Within groups	10,538.508	36	292.736		
Total	60,059.637	39			

**Table 4:** Comparison of mean enamel surface microhardness after remineralization between different groups

Groups	Mean difference	Std. error	p-value
Control $\frac{1}{5}$ GC Tooth mousse	36.8533	7.6516	0.000
Control $\frac{1}{5}$ Acclaim	65.5733	7.6516	0.000
Control $\frac{1}{5}$ EnaFix	95.1466	7.6516	0.000
GC Tooth mousse $\frac{1}{5}$ Acclaim	28.7200	7.6516	0.003
GC Tooth mousse $\frac{1}{5}$ EnaFix	58.2933	7.6516	0.000
Acclaim $\frac{1}{5}$ EnaFix	29.5733	7.6516	0.002

In the control group the increase was  $6.34 \pm 6.34$  KHN whereas, in the other groups the increase ranged from  $39.91 \pm 22.76$  to  $96.28 \pm 14.4$  KHN. The increase in enamel surface microhardness observed was highest ( $96.28 \pm 14.40$ ) in group IV (EnaFix™) compared to group II (GC Tooth Mousse™) ( $39.91 \pm 26.76$ ) and group III (Acclaim™) ( $52.85 \pm 18.53$ ). A study by De Carvalho FG et al.<sup>9</sup> comparing enamel microhardness of CPP-ACP and nano-(HAP) toothpaste shows a significant increase in microhardness in nano-(HAP) compared to CPP-ACP, however, both toothpastes show an increase in baseline hardness value after remineralization which was similar to our study. Latha S et al.<sup>10</sup> and Zhang Q et al.<sup>8</sup> also observed an increase in enamel surface microhardness after remineralization with CCP-ACP cream. The %SMHR range from 1.72% in group I (control) to 25.90% in group IV. EnaFix™ (Calcium Sucrose Phosphate) toothpaste showed a greater %SMHR when compared to Tooth Mousse™ (CCP-ACP) and Acclaim™(nano-(HAP)) toothpaste. Zhang Q et al.<sup>8</sup> also reported an increase in %SMHR by 6.13% in the control group and 73.7% in CPP-ACP group. The increase was higher in comparison to the results in our study, which could be due to the better stabilization of minerals in the enamel crystal latex of permanent teeth compared to primary teeth. Similarly, de Carvalho FG et al.<sup>9</sup> also observed an increase of 11% in %SMHR with CPP-ACP paste which was similar to our observation. In nano-(HAP) group the %SHMR was 57.9% whereas it was 14.52% in our study. It is due to the existence of 9000 ppm fluoride along with hydroxyapatite paste. As there were no studies, which reported the % SMHR with EnaFix™ (Calcium Sucrose Phosphate), a comparison was not possible. However, Thomas RR and Acharya SR<sup>13</sup> reported a higher increase in enamel surface microhardness when compared to CPP-ACP group and it was observed that there was a maximum increase in enamel surface microhardness in the EnaFix™ (Calcium Sucrose Phosphate) group when compared to all the other groups. Similarly, Acclaim™ (nano-HAP) group had higher enamel surface microhardness compared to GC Tooth Mousse™ (CPP-ACP). Although various studies<sup>8-10,14</sup> have reported an increase in enamel surface microhardness with CPP-ACP paste, in comparison with nano-(HAP) and Calcium Sucrose Phosphate the difference was less. Similarly, the remineralization potential of CPP-ACP paste was also reported by other investigators<sup>15-17</sup> using different methods.

For the remineralization process to occur with CPP-ACP paste, natural saliva and acquired pellicle are required for better retention on the tooth surface. It is assumed that under oral conditions Casein phosphopeptide can bind to biofilm and act as a pool of slow-releasing calcium and phosphate ions,<sup>18</sup> this complex oral environment is difficult to replace in an *in vitro* study, which probably leads to the decrease in remineralization potential of CPP-ACP.

The remineralization effect of nano-(HAP) on caries lesions was reported by various investigators.<sup>19-22</sup> Huang S et al.<sup>11</sup> proposed that the increased remineralization potential of hydroxyapatite is due to its calcium phosphate reservoir which helps to maintain a state of supersaturation with respect to enamel minerals. In our study, nano-(HAP) increases the enamel surface microhardness significantly indicating remineralization.

Calcium sucrose phosphate toothpaste has been reported to have remineralization potential in studies conducted by Sargod SS et al.<sup>23</sup> and Thomas RR and Acharya SR.<sup>13</sup> In the present study EnaFix™ (calcium sucrose phosphate) exhibited the maximum increase in enamel remineralization when compared to the other agents. Calcium and phosphate in aqueous media tend to form insoluble precipitates, which decreases the rate of dissolution of hydroxylapatite in the enamel.

## CONCLUSION

Based on the observations of our study, the following conclusions were drawn:

- GC Tooth Mousse™ (CPP-ACP), Acclaim™ (nano-HAP), and EnaFix™ (Calcium Sucrose Phosphate) toothpaste show an increase in the enamel surface microhardness values.
- EnaFix™ (Calcium Sucrose Phosphate) shows a maximum increase in the enamel surface microhardness followed by Acclaim™ (nano-HAP) and GC Tooth Mousse™ (CPP-ACP) toothpaste.

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