# Use of Laser fluorescence and Scanning Electron Microscope to Evaluate Remineralization of Incipient Enamel Lesions Remineralized by Topical Application of Casein Phospho Peptide Amorphous Calcium Phosphate (CPP-ACP) Containing Cream

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*The aim of this in vitro study was to evaluate the remineralization of incipient enamel lesions by the topical application of Casein PhosphoPeptide-Amorphous Calcium Phosphate (CPP-ACP) using laser fluorescence and scanning electron microscope.*

*Sixty caries free extracted teeth were used in the study. Forty teeth were used as test samples, ten as positive and ten as negative controls. The samples were demineralized and then remineralized by the topical application of CPP-ACP for a period of 14 days. The remineralization was evaluated with the use of laser fluorescence and scanning electron microscope (SEM). The results of this study showed that the laser fluorescent readings of test samples after remineralization were highly significant (p <0.001). A significant number of test samples observed under SEM showed high scores of remineralization.*

***Keywords:*** *Casein PhosphoPeptide-Amorphous Calcium Phosphate (CPP-ACP), Remineralization, Laser fluorescence.*

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## INTRODUCTION

The enamel of teeth is a porous structure that allows the access of ions into its deeper layers. The presence of an electrically charged hydration layer around the enamel crystals is known to be responsible for the capacity of enamel to remineralize non cavitated lesion with minimal intervention.[1] A number of methods and agents are available to remineralize early enamel carious lesions. The topical application of mineral-rich concentrates can also be used for remineralization of such incipient carious lesions. This provides calcium and phosphate ions that can easily diffuse through porous enamel in order to remineralize incipient enamel lesions.[2,3]

The use of mineral rich concentrates (like that in GC Tooth mousse) derived from milk and milk products to remineralize carious lesions is not a new concept. The influence of milk and its derivatives on caries is known since the 1950's, when cheese was considered to have a substantial cariostatic effect. Although it was attributed to the physical nature of cheese and the presence of casein, calcium and phosphate contents.[4] Later investigations on the role of casein and calcium phosphate concentrates derived from milk on remineralization concluded that casein phosphopeptide complexes stabilized the calcium phosphates and remineralized the incipient carious lesions.[5,6] Since then the efforts to halt the progression of caries or even revert the disease in its early stages with the use of CPP- ACP have been phenomenal. CPP-ACP is made available as an active component in chewing gums, lozenges, topical creams that are used clinically for anticariogenity and cariostasis.

When such an agent is used clinically the practitioner

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should evaluate the remineralization with the use of non-invasive techniques. Therefore this *in vitro* study was designed to use fluorescent device to evaluate the remineralization of incipient enamel lesions remineralized using CPP-ACP containing topical cream. The remineralization was also evaluated using scanning electron microscope (SEM) to confirm the observations made by the laser fluorescent device.

## MATERIALS AND METHODS

The ethical research committee of the Yenepoya Medical and Dental College, Mangalore, India, approved the study.

Sixty caries free premolar and molar teeth requiring extraction for orthodontic reasons and impactions, without any visible or detectable caries, hypoplastic lesions, stains (extrinsic or intrinsic) and white spot lesions on any surface of the tooth, were selected.

These teeth were then sectioned mesiodistally with the help of a diamond disc 0.15mm thickness and 22mm diameter (Dentaurum, Germany) mounted on a straight slow-speed handpiece with water coolant. The sections were painted with an acid resistant nail varnish exposing a window of 2 x 2 mm on the center of the buccal or lingual surfaces. The samples were allowed to dry and then divided into three groups randomly. Forty sections were used as test samples, ten served as positive control and ten served as negative controls.

The baseline laser fluorescent readings within the exposed window of all samples were recorded using the Kavo DIAGNOdent 2095 {Best nr-5740500,Kaltenbach and Voigt Gmb and Co, Biberach}. The laser fluorescence readings were performed using tip B probe. The device was calibrated against a porcelain reference object prior to examination and recalibrated after reading ten teeth. The peak values of the samples were recorded.

The teeth were then demineralized. The demineralizing solution was prepared by adding 1% of sodium carboxymethylcellulose to 0.1 M Lactic acid containing 3mM of calcium and 1.8mM of phosphate and the pH was adjusted to 4. The sections of teeth were then immersed in the demineralization solution so prepared, at 37 degree Celsius, in an incubator for a period of sixteen hours.[7] The laser fluorescent device was used to evaluate the presence of demineralization using the cut-off points recommended by the manufacturer.[8]

The presence of demineralization was also confirmed by observing the surface morphologic changes under scanning electron microscope {JEOL JSM 840ASM, JAPAN}.

The enamel samples were prepared for observation of surface morphology under scanning electron microscopy. The samples were attached to aluminum stubs and then sputtered water with gold using the fine gold sputtering unit under vacuum. The surface morphology of these samples was then examined under SEM (JEOL JSM 840ASM, JAPAN) and studied at 20 KV and photographed at 30X, 500X and1000X magnification. The interpretation of the SEM observations showed varying degree and extent of demineralization varying from cratered, eroded and typical honey comb appearance as described in previous reports.[9,10]

The test samples were then remineralized by a daily application of GC Tooth mousse (GC Corporation, Japan) that contains CPP-ACP as the active ingredient for a period of fourteen days as recommended by the manufacturer. The GC Tooth mousse was applied onto the tooth surface window with a brush and left in place for three minutes. The samples were then immersed in artificial saliva[11] and incubated at 37 degrees Celsius. The artificial saliva was changed every day.

The positive control samples were left in artificial saliva during the test period. The negative control samples were left in normal saline during the test period.

All the samples were evaluated for remineralization using the laser fluorescent device and SEM.

The samples were considered remineralized when digital readings of laser fluorescence increased when compared with the demineralized readings.

Scores were assigned depending on the pattern of remineralization as observed in a previous report.[12]

The data obtained from the test samples from both laser fluorescence and SEM observations were subjected to statistical analysis.

## RESULTS

A Change in fluorescence of test samples from demineralization to remineralization indicated that a significant amount of remineralization had taken place in these samples. The surface morphologic changes of all test samples showed evidence of remineralization as observed under SEM.

The digital readings of the changes in fluorescence from Baseline-demineralization- remineralization of all the samples of test group, positive and negative control were statistically analyzed as shown in Tables 1 and 2.

The statistical analysis showed that changes in fluorescence from demineralization to remineralization in the test group were highly significant ($p<0.001$). The changes in fluorescence of samples in the positive control from demineralization to remineralization were not statistically

**Table 1.** Comparison Of Diagnodent Readings Between Test Group, Positive And Negative Control by Mann Whitney U test

| | N | Mean | Std Deviation | P Value |
|---|---|---|---|---|
| **Baseline** | | | | |
| Test | 40 | 7.7075 | .7770 | .061- |
| Positive control | 10 | 7.2000 | .2708 | |
| Negative control | 10 | 7.3900 | .3071 | |
| Demineralization | | | | |
| Test | 40 | 12.0750 | .8883 | .177- |
| Positive control | 10 | 12.2000 | .3496 | |
| Negative control | 10 | 11.7500 | .6346 | |
| Remineralization | | | | |
| Test | 40 | 8.4750 | .8767 | .001** |
| Positive control | 10 | 11.7000 | .6749 | |
| Negative control | 10 | 11.6000 | .8433 | |

**Table 2.** Comparison Of The Diagnodent Readings Within Each Group using Wilcoxon signed rank sum test

| | Paired differences | | | |
|---|---|---|---|---|
| GROUP | Mean | Std. Deviation | Z | P |
| Test<br>Baseline-demineralization<br>Demineralization-remineralization | <br>-4.3675<br>3.6000 | <br>1.2827<br>1.3359 | <br>5.542<br>5.479 | <br>0.001**<br>0.001** |
| Positive control<br>Baseline-demineralization<br>Demineralization-remineralization | <br>-5.0000<br>0.5000 | <br>0.5011<br>0.5774 | <br>2.812<br>1.839 | <br>0.005<br>0.062 - |
| Negative control<br>Baseline-demineralization<br>Demineralization–remineralization | <br>-4.3600<br>0.1500 | <br>0.7545<br>1.2030 | <br>2.870<br>0.184 | <br>0.005<br>0.854 - |

• = highly significant, ** =very highly significant,
- = not significant

**Table 3.** Comparision of Scanning electron microscopoic observation between the groups

| | **GROUP** | | | **TOTAL** |
|---|---|---|---|---|
| | **TEST** | **POSITIVE** | **NEGATIVE** | |
| SCORE 0<br>Count % | | 7<br>(70%) | 10<br>(100%) | **17**<br>**(28.3%)** |
| SCORE 1<br>Count % | 8<br>(20%) | 3<br>(30%) | | **11**<br>**(18.3%)** |
| SCORE 2<br>Count % | | | | |
| SCORE 3<br>Count % | 10<br>(25%) | | | **10**<br>**(16.7%)** |
| SCORE 4<br>Count % | 22<br>(55%) | | | **22**<br>**(36.7%)** |
| SCORE 5<br>Count % | | | | |
| **TOTAL**<br>**Count %** | **40**<br>**(100%)** | **10**<br>**(100%)** | **10**<br>**(100%)** | **60**<br>**(100%)** |

Chi square Value = 54.225; p=0.001vhs

significant (p value > 0.05). The samples in the negative control did not exhibit statistically significant changes in fluorescence from demineralization to remineralization.

It was therefore interpreted that the samples of the test groups showed significant remineralization as compared to other groups.

SEM results were statistically analyzed as depicted in Table 3. Twenty two (55 %) of the test samples showed significant remineralization corresponding to score 4. 10 (25 %) of the samples were assigned score 3 of remineralization, 8 (20%) of the samples exhibited remineralization corresponding to score 1.

In the positive control group 7 (70%) samples did not remineralize where as the observations of 3 samples (30%) correlated to score1. In the negative control none of the samples remineralized.

## DISCUSSION

Changing patterns of dental caries are not only attributed to preventive approaches but also to the early interceptive strategies employed.[13] Early interception include early detection and remineralization of incipient lesions. This is made possible by the use of sensitive noninvasive techniques like the laser fluorescent devices and the use of remineralizing agents like CPP-ACP, which is derived from milk concentrate.

The caries reducing effect of dairy products is attributed to prevention of demineralization and /or remineralization brought by casein, ionizable calcium and phosphate species present in the dairy foods.[2,4,15,16,17]

The role of casein forms a protective coat on the enamel surface and prevents demineralization. The calcium and phosphate complexes bring about the remineralization. The caseinate protein resisted the proteolytic action of the enzymes and continues to stabilize calcium phosphate complexes favoring remineralization.[18]

These casein phosphopeptide (CPP) contain a cluster of phosphoseryl residues in the motif–Ser(P)–Ser(P)-Ser(P)-Glu-Glu- which markedly increases the apparent solubility of calcium phosphate by stabilizing amorphous calcium phosphate (ACP) under both neutral and alkaline conditions, forming metastable solutions that are supersaturated with respect to the calcium phosphates in plaque.[3,7,14,19,20,21] The proposed mechanism is that CPP-ACP acts as a calcium reservoir, buffering the activities of free calcium phosphate ions in the plaque fluid helping to maintain a state of super saturation with respect to enamel mineral, there by depressing enamel demineralization and enhancing remineralization.[14,20]

In this *in vitro* study, CPP-ACP containing cream produced significant amount of remineralization as evaluated with laser fluorescence and SEM and is in agreement with earlier reports.[5,18,20]

When the samples were evaluated with the laser fluorescence device, it was found that the test samples showed a significant change in their fluorescent readings from demineralized state to the end of fourteen days of test period of remineralization. This observation was in agreement with earlier reports on the use of laser fluorescent device to monitor caries progression and efficiency of preventive regimens both for natural and simulated caries lesions.[22, 23,24,25,26,27]

Alterations in the mineral content of enamel as in the demineralization and remineralization process directly brings about change in optical properties, including fluorescence.[25,28] Therefore change in laser fluorescence has been used to monitor changes in mineral content associated with both demineralization and remineralization.[24,25,26,27]

This change in optical properties was interpreted in this study in terms of change in the laser fluorescent readings of the samples from baseline to loss of minerals as demineralization and gain in minerals corresponding to remineralization.

The SEM was also used to evaluate the remineralization. Hence few studies are available regarding its use in detecting

the early changes in mineral loss and its correlation to caries progression or remineralization. Also the mechanism responsible for the change in fluorescence in carious lesion is not yet clearly established. Since some reports claim that fluorescence in carious lesions can be detected only with presence of bacterial components. The change in fluorescence is related to porphyrins produced by the bacterial action. The other school of thought explains that the increase in tissue porosity with respect to carious process is responsible for the change in fluorescence.[23]

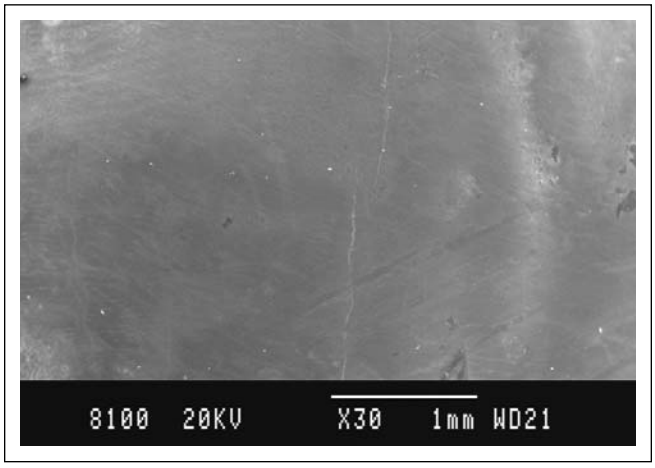


**Figure 1a.** Photomicrograph of enamel sample number 1 showing remineralization at 30X magnification as observed under scanning electron microscope.

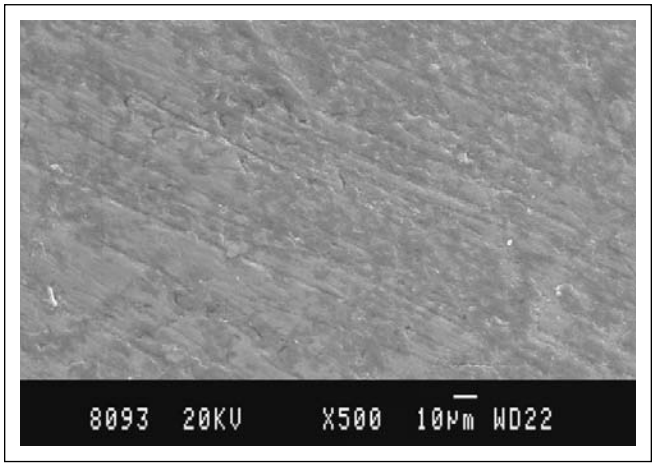


**Figure 1b.** Photomicrograph of enamel sample number 1 showing remineralization at 500X magnification as observed under scanning electron microscope.

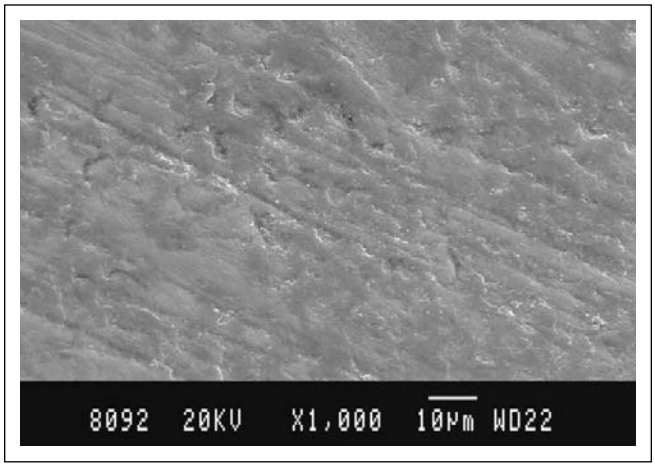


**Figure 1c.** Photomicrograph of enamel sample number 1 showing remineralization at 1000X magnification as observed under scanning electron microscope.

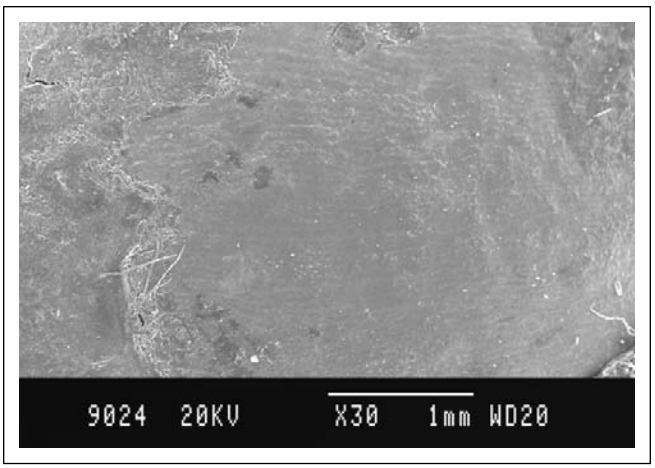


**Figure 2a.** Photomicrograph of enamel sample number 2 showing remineralization at 30X magnification as observed under scanning electron microscope.

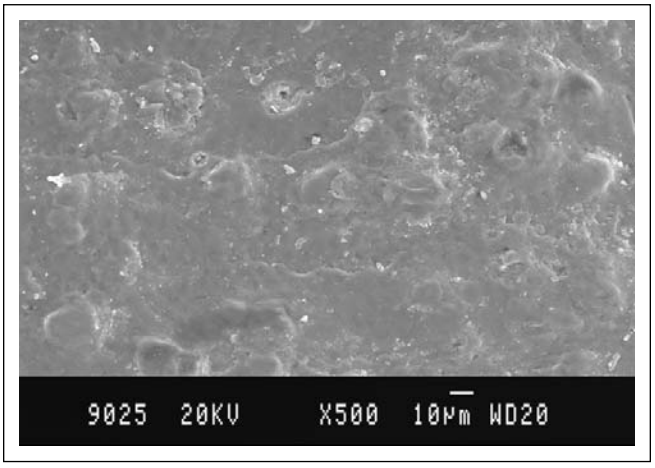


**Figure 2b.** Photomicrograph of enamel sample number 2 showing remineralization at 500X magnification as observed under scanning electron microscope.

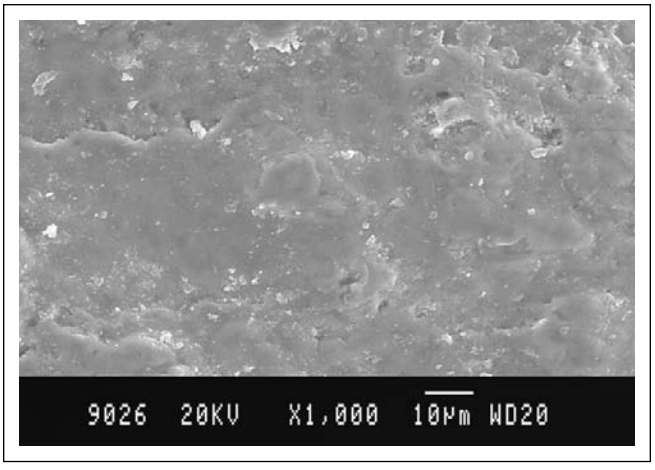


**Figure 2c.** Photomicrograph of enamel sample number 2 showing remineralization at 1000X magnification as observed under scanning electron microscope.

SEM is one of the most sensitive, time-tested techniques to assess the demineralization and remineralization of the carious lesions *in vitro* as reported in earlier studies.[10,11,29,30]

The samples in this study were also evaluated for remineralization using the SEM.[12] The surface topography of the test samples showed varying degree and extent of remineralization and is in agreement with earlier reports.[29,30,31]

In this study both laser fluorescence and SEM were used but no comparison between the two was considered, since laser fluorescence quantitatively evaluates the changes in fluorescence properties of enamel from baseline, after demineralization and after remineralization whereas the SEM observation of the surface morphology is a qualitative analysis of enamel.

This study was conducted simulating a few intraoral conditions under an *in vitro* model to demonstrate the remineralization of simulated carious lesions. The replication of the dynamics of the caries process and the complexity of the oral environment in these *in vitro* models is limited. The effect of the remineralizing potential of natural saliva, the cyclic changes during the demineralization and remineralization and the effect of bacterial assaults in a clinical situation were not determined.

CPP-ACP binds to dental plaque, and is able to slow or prevent the diffusion of calcium ions from enamel during episodes of acid challenge and act as a reservoir of calcium ions for subsequent remineralization. Hence further studies to evaluate the ability of CPP-ACP to remineralize lesions under acidic condition *in vivo* are essential.

Studies that can equate to the amount of mineral loss or gain quantitatively to change in fluorescent readings, in order to establish appropriate cut-off points to monitor the progress of demineralization and remineralization are required. This can make it more convenient to set clinical standards for use of laser fluorescence as a chair side method.

Given the limitations of *in vitro* studies and the dynamics of *in vivo* conditions, *in vivo* studies that evaluate the remineralization of CPP-ACP with the use of suitable devices like laser fluorescence can validate the remineralizing potential of CPP-ACP in clinical situations.

## CONCLUSIONS

CPP-ACP being a mineral rich concentrate, when complexed with protein like casein acts as a reservoir and has substantial buffering capacities. Hence CPP-ACP can prevent demineralization and also bring about remineralization in enamel lesions. In this *in vitro* study the topical application of CPP-ACP containing cream on demineralized enamel exhibited significant remineralization as evaluated by laser fluorescence and scanning electron microscopy.

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