ORIGINAL RESEARCH

# Assessment of Remineralization Capacity of Various Remineralizing Agents on Artificial Enamel Lesions Using Confocal Laser Scanning Microscope: An *In Vitro* Study

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## Abstract

**Aim and objective:** The present study aimed at evaluating the effectiveness of diverse remineralizing agents on artificial enamel lesion using confocal laser scanning microscope (CLSM).

**Materials and methods:** Totally 80 mandibular premolars which were single rooted were included. All teeth were suspended in a demineralizing solution to create artificial enamel lesions on the exposed enamel. The samples were separated randomly into four groups (20 each) depending on the application of the remineralizing agents as follows: group 1: control; group 2: calcium sucrose phosphate (CaSP); group 3: fluoride varnish; and group 4: casein phosphopeptides-amorphous calcium phosphate (CPP–ACP). The samples in individual group were treated with the corresponding remineralizing agent (except for the control group) two times a day for 14 days. The experimental and control groups were exposed to CLSM assessment to analyze the data of remineralization and demineralization.

**Results:** The mean depth of remineralization of fluoride varnish group was slightly more compared to other groups. The highest mean depth of remineralization was found in the fluoride varnish group (122.26 ± 0.28) followed by CaSP (110.58 ± 1.34), CPP–ACP (107.08 ± 0.48), and control (157.78 ± 0.46) groups. The different comparisons among the remineralization material groups showed a statistically significant difference ($p < 0.05$) in almost all groups except group 2 vs group 4.

**Conclusion:** This study concluded that improved remineralization of artificial enamel lesion could be achieved with the fluoride varnish group when compared to the CaSP and CPP–ACP groups.

**Clinical significance:** Remineralization as a treatment technique has received a lot of consideration from clinicians. The process of remineralization and demineralization is considered an active process categorized by the movement of calcium and phosphate in and out of the enamel. Presently, the attention has changed toward increasing the resistance of the tooth by applying remineralizing agents topically, which has led to the notable fall in dental caries.

**Keywords:** Confocal laser scanning fluorescence microscopy, Demineralization, Remineralizing agents, Rhodamine B.

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## Introduction

Dental caries is an enduring microbial infection of the calcified tissues of the teeth portrayed by demineralization of the inorganic part and devastation of the natural substance of the tooth, which regularly prompts cavitation. Demineralization is the atypical loss of surface or subsurface mineral components of enamel at reduced salivary pH, below the "critical pH," caused due to organic acids produced by caries-causing bacteria. The process of recovery of new minerals from the oral structures and the natural processes to facilitate remineralization is the chief objective. Calcium and phosphorus are the main mineral components of teeth. The process of sourcing new constituents or mineral components to enable teeth remineralization has been the main stay for the present research.[1]

The best effective way to avoid decay of tooth is to remineralize the tooth before formation of a cavity in its most initial and incipient stage. The remineralizing agents provide the required ions by generating some surface coatings which perform as diffusion blocks and thus reduce the solubility of the enamel by mineral deposition within the enamel crystals. The World Health Organization expert group reported a reduction in the prevalence rates of dental caries across several countries and this was ascribed to the extensive use of remineralizing agents.[2]

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Fluoride is a highly recognized remineralizing agent which intermingles with the oral liquids present on the outer layer of the enamel and the subsurface regions of teeth, compounding with the phosphate and calcium particles to shape fluorapatite. The advantages of fluoride as an anticaries substance are dependent on its use in an efficacious concentration and rate of application.[3]

The oral care products containing CPP–ACP when applied in an *in situ* environment are shown to remineralize the subsurface lesions of the enamel. The possible effect of CPP–ACP as an anticariogenic agent is its ability to confine ACP along the tooth's surface, which causes buffering of phosphate- and calcium-free ion movement, thus facilitating the maintenance of a supersaturation condition with regard to the tooth enamel preventing demineralization and promoting remineralization.[4]

A calcium sucrose phosphate (CaSP)–calcium orthophosphate complex provides both phosphate and calcium in a solvable form. It is a white, fine, nonhygroscopic powder with a neutral blend taste. It comprises around 11.5% calcium on a dry weight basis. This compound helps minimize the solubility of the enamel by an acid and enhances the remineralization rate due to common ion effect. CLSM is a ground-breaking imaging strategy depending on the optical conduct of light inside the samples. For the most part, confocal examination highlights excitation (e.g., fluorescence) despite the fact that emanation identification is additionally conceivable. The optical property of this technique helps in the identification of carious and noncarious lesions of the tooth.[5] Until this point, very little data are accessible on the utilization of a confocal microscope for the investigation of early enamel lesions of the tooth and several procedures have been adapted to achieve remineralization of incipient caries of the enamel. After accounting these parameters, this *in vitro* research was done to assess the remineralizing ability of various remineralizing agents on artificial enamel lesions supported by a CLSM.

## Materials and Methods

This *in vitro* research was done in the Department of Conservative Dentistry and Endodontics, Buddha Institute of Dental Sciences and Hospital, Patna, Bihar. Totally 80 lower premolars having single root which were extracted for orthodontic reasons from patients aged 18–25 were included. Teeth which were noncarious, morphologically intact, and free of discoloration and hypoplasia were included in this study. Carious teeth and teeth with visible cracks, attrition, restorations, and developmental anomalies were excluded from the study. After sterilization, all teeth were stored in 1% thymol solution until additional preparation.

### Enamel Window Preparation

All the teeth were polished on the buccal surfaces and a 1-mm cut was made 1 mm below the cementoenamel junction. The teeth were later implanted in the acrylic blocks so that crown portions were seen. A zone of 4 mm × 4 mm (window) was marked on the buccal surfaces of all teeth, glazed with a nail varnish (Colorama nail varnish, Maybelline) excluding the window, which was examined for the variation of fluorescence values after demineralization and remineralization.

### Artificial Enamel Lesion Preparation

Enamel lesions were artificially made on the uncovered polish by drenching all the teeth in a demineralizing solution, a combination of trisodium phosphate [2.0 mmol/L] and calcium chloride [2.0 mmol/L] in a buffer solution of acetate [75 mmol/L] for four days at a pH of 4.6.[6] Recently, acids were being used to create caries-like lesions and this has replaced the use of acids produced by the microorganisms. These processes have further enhanced the understanding of the demineralization and remineralization processes. The samples were divided randomly into four groups (20 each) based on the application of the remineralizing agents after four days as follows:

Group 1: Control (no remineralizing agent)
Group 2: CaSP (Toothmin paste, Abbott Healthcare, Mumbai, India)
Group 3: Fluoride varnish (Fluor Protector, Ivoclar Vivadent,)
Group 4: CPP–ACP (Recaldent GC Tooth Mousse, GC Corp, Japan)

### Preparation of Artificial Saliva and Remineralization Process

Gastric mucin (2.2 g/L) was mixed with sodium chloride (0.381 g/L), potassium hydrogen phosphate (1.114 g/L), and calcium chloride (0.213 g/L) to make artificial saliva.[7] The ingredients were added individually to deionized water with constant stirring and were allowed to dissolve completely before the next ingredient was added. The solution was stored at 37°C and 85% lactic acid was used to adjust the pH to 7. The samples were then treated with the corresponding remineralizing agent (excluding the control group) two times a day for 14 days for 3 minutes with the help of a cotton applicator tip. The samples were then washed with distilled water and stored in artificial saliva. The samples in the control group were washed with distilled water alone and kept in artificial saliva which was restored every day.

### Confocal Laser Scanning Microscopic (CLSM) Evaluation

The control and experimental group were exposed to evaluation by CLSM so as to assess the baseline data of remineralization and demineralization. The teeth samples from the experimental and control groups were stored in rhodamine B dye (1 mM) for a day and then sliced and visualized under the confocal microscope.

The sections which were stained were fixed and modeling wax was used to mount on the microscope glass slide. Argon laser at half force was created utilizing a 488 nm excitation frequency and was used to illuminate using a 10x objective. Confocal cuts were set up at 25 µm with a 515-nm-long-pass channel. The areas planoparallel to the specimen's cut surface were scanned.

The CLSM images (Figs 1 and 2) were scored separately by another examiner who was blinded to sample groups using the following criteria. The sound enamel (untreated specimen) appeared pitch black near 0 fluorescence (grayscale value approximately 0) registers. Lesions (demineralized specimen) were somewhat auto fluorescent; be that as it may, the retention of the rhodamine dye (0.1 mM) permitted the demineralized permeable layer to fill and show up with significant differentiation (higher gray values). Remineralized tests showed a diminished fluorescence (lesser gray values which are demonstrative of decreased porosity and entrance of dye or more minerals).

The decrease in the fluorescence (gray values) was connected between the three groups to assess the remineralization capability of each agent.

### Statistical Analysis

SPSS software (version 20.0) was utilized to figure the mean and standard deviation. One-way analysis of variance (ANOVA) was

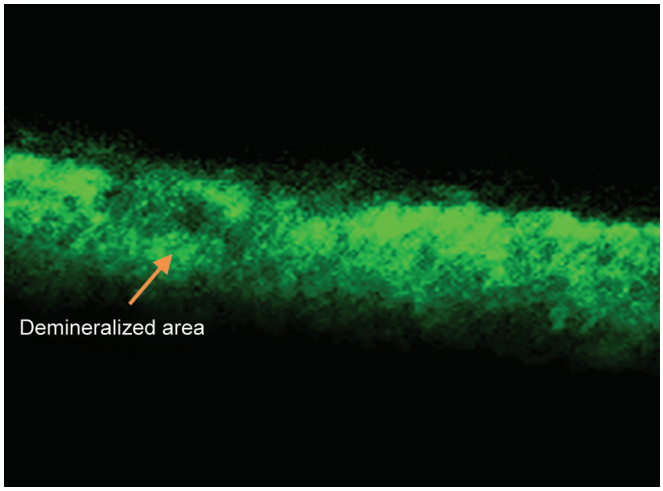


**Fig. 1:** Confocal laser scanning microscopic image after artificial enamel lesion preparation

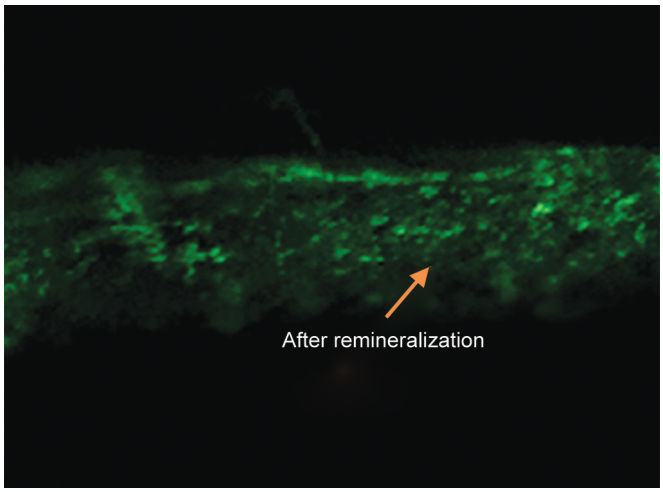


**Fig. 2:** Confocal laser scanning microscopic image after remineralization of enamel lesion

used to compare between the different groups of remineralization agents. A *p*-value estimation of under 0.05 was considered statistically significant.

## Results

Table 1 displays the comparison of the mean depth of remineralization among different study groups. The fluoride varnish group's mean depth of remineralization was a little more (160.74 ± 1.07) followed by CaSP (159.23 ± 1.01), CPP–ACP (158.43 ± 0.26), and control (157.78 ± 0.46) groups. There were no significant differences between the study groups.

The comparison of the mean depth of remineralization among different study groups is as shown in Table 2. The highest mean depth of remineralization was found in the fluoride varnish group (122.26 ± 0.28) followed by CaSP (110.58 ± 1.34), CPP–ACP (107.08 ± 0.48), and control (157.78 ± 0.46) groups. There was a statistically significant difference in the remineralization between the three experimental groups.

There was increased depth of demineralized zone remaining after remineralization of the CPP–ACP group (51.35 ± 0.22) followed by CaSP (48.65 ± 0.33) and fluoride varnish (38.48 ± 0.79) groups. A significant difference was found between the remineralizing agents (Table 3).

Multiple evaluations among the remineralization material groups are as displayed in Table 4. A statistically significant difference ($p < 0.05$) was found in all groups except group 2 vs group 4.

**Table 1:** Comparison of the mean depth of demineralization among different study groups

| *Groups* | *N* | *Mean ± std. deviation (μ)* | *F* | *p-value* |
|---|---|---|---|---|
| Group 1: Control | 20 | 157.78 ± 0.46 | 27.196 | 0.388 |
| Group 2: CaSP | 20 | 159.23 ± 1.01 | | |
| Group 3: Fluoride varnish | 20 | 160.74 ± 1.07 | | |
| Group 4: CPP–ACP | 20 | 158.43 ± 0.26 | | |

**Table 2:** Comparison of the mean depth of remineralization among different study groups

| *Groups* | *N* | *Mean ± std. deviation (μ)* | *F* | *p-value* |
|---|---|---|---|---|
| Group 1: Control | 20 | 157.78 ± 0.46 | | |
| Group 2: CaSP | 20 | 110.58 ± 1.34 | | |
| Group 3: Fluoride varnish | 20 | 122.26 ± 0.28 | | |
| Group 4:CPP–ACP | 20 | 107.08 ± 0.48 | 24.226 | 0.001 |

## Discussion

Recently, the attention to caries treatment has moved toward advancement of the processes for the identification of the incipient phases of carious lesions and their treatment noninvasively. The remineralization method of treating early lesions noninvasively has a possibility of providing major progress in the clinical management of the disease. The remineralization of early, white-spot caries can likely be achieved with several presently available agents.[8]

Different quantitative and qualitative methods that measure changes in tooth mineral content, such as the use of light scattering, transverse microradiography, energy-dispersive X-ray analysis, polarized light microscopy, polarization-sensitive optical coherence tomography, CLSM, and cross-sectional microhardness determination, have been used to analyze the caries remineralization potential of various remineralizing agents. CLSM is an important imaging procedure depending on the optical performance of light inside the specimens. Even though detection of emission is possible with confocal analysis, it even features excitation (such as fluorescence). This optical property benefits the recognition of carious and noncarious lesions of the teeth.[9]

In this study, fluoride varnish group was significantly found to be a better remineralizing agent compared to the CaSP and CPP–ACP groups. Similar to these results, Shirahatti et al.[10], reported a considerable protective effect of fluoride-containing agents against formation and progression of a lesion. But, CPP–ACP paste did not have any added capability of decreasing the development of lesion depth.

**Table 3:** Overall depth of demineralized area after remineralization among all groups

| *Groups* | *Depth of demineralized area (before) (μ)* | *Depth of remineralized area (μ)* | *Depth of demineralized area after remineralization (μ)* | *F* | *p-value* |
|---|---|---|---|---|---|
| Group 1: Control | 157.78 ± 0.46 | 157.78 ± 0.46 | 0 | 2.874 | 0.001 |
| Group 2: CaSP | 159.23 ± 1.01 | 110.58 ± 1.34 | 48.65 ± 0.33 | | |
| Group 3: Fluoride varnish | 160.74 ± 1.07 | 122.26 ± 0.28 | 38.48 ± 0.79 | | |
| Group 4: CPP–ACP | 158.43 ± 0.26 | 107.08 ± 0.48 | 51.35 ± 0.22 | | |

**Table 4:** Comparisons of depth of remineralization using Tukey HSD

| *Group* | *Compared with* | *Mean difference (I-J)* | *Sig.* |
|---|---|---|---|
| Group 1 | Group 2 | 47.2 | 0.001 |
| | Group 3 | 35.52 | 0.001 |
| | Group 4 | 50.7 | 0.001 |
| Group 2 | Group 1 | −47.2 | 0.001 |
| | Group 3 | −11.68 | 0.001 |
| | Group 4 | 3.5 | 0.09 |
| Group 3 | Group 1 | −35.52 | 0.001 |
| | Group 2 | 11.68 | 0.001 |
| | Group 4 | 15.18 | 0.001 |
| Group 4 | Group 1 | −50.7 | 0.001 |
| | Group 2 | −3.5 | 0.09 |
| | Group 3 | −15.18 | 0.001 |

Similarly, Reynolds et al.[11] demonstrated a marginal increase in remineralization in the fluoride group compared to that of the CPP–ACP group. A significant difference between the groups was shown by the one-way ANOVA analysis.

Our results are not in accordance with the results obtained by Divyapriya et al.[12] and Lata et al.[13]. According to them, casein phosphopeptides present in the CPP–ACP comprise multiphosphoseryl sequences, which can localize and stabilize ACP at the tooth surface when applied on the enamel thereby enhancing the levels of calcium and phosphate in the plaques that serve as a pool of calcium phosphate. These increased saturation levels with regard to the tooth enamel resulting in the formation of firm crystalline phases, such as apatite products or octacalcium phosphate, which reduces demineralization of the enamel and increases its remineralization.

In this study, better remineralization results were demonstrated by the CaSP group than the CPP–ACP group. It was concluded by Menon et al.[14] that calcium levels in saliva are significantly increased by CaSP resulting in increased deposition of calcium in the tooth structures. In addition to increased calcium deposition in the tooth structures, CaSP also reduces the plaque deposition in the tooth surfaces. As per Gangrade et al.[15] study, the remineralizing agents did not show any statistically significant differences in the remineralizing efficacy even though the CaSP group showed a greater mean microhardness value than the CPP–ACP group, which was in accordance with the results obtained by this study. The remineralization potential of different materials was evaluated by Kaur et al.[16] with the help of surface microhardness test. They reported that there was no significant difference in the remineralizing effectiveness of CPP–ACPF and CaSP while a slightly increased rate of remineralization potential was shown by CaSP than CPP–ACPF.

The reprecipitation and dissolution process along the oral fluid-tooth interface is altered continuously by fluoride present intraorally. Minimal amounts of fluoride can accelerate remineralization of early-stage caries.[17] With the use of high-concentration fluoride treatment, calcium fluoride aggregates deposits on the tooth surfaces and these later act as a fluoride reservoir. At low pH, the rate of fluoride release is increased.[18]

Fluoride varnish was shown to be significantly better than the CPP–ACP group. The presence of microorganisms, plaque, and saliva present in the *in situ* state fundamentally improves the cycle of remineralization which would not be conceivable in an *in vitro* condition. This could probably be the explanation behind the fluoride varnish group to be superior to the CPP–ACP group. The results obtained by Chokshi et al.[3] are similar to our study results and they recorded the greatest remineralization potential with the use of fluoride varnish in artificial carious lesions followed next by CPP–ACP paste.

In the present study, the fluoride varnish agent significantly depicted as a better remineralization agent on artificial enamel lesions. The limitation of this study is that the duration of remineralization used in this study was two weeks and during this period artificial caries could not be remineralized completely, thus the duration of application of remineralizing agents used in this study cannot be defined for total remineralization. This study confirmed surface remineralization; however, subsurface remineralization of the enamel was not assessed in this study. One must always remember that remineralization could be pretty different *in vitro* when compared with the *in vivo* active compound biological system which occurs intraorally. Therefore, drawing inferences from *in vitro* clinical conditions must be followed with caution because of obvious drawbacks of such studies.

## Conclusion

This study concluded that improved remineralization of artificial enamel lesions could be achieved with the fluoride varnish group when compared to the CaSP and CPP–ACP groups.

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