# Comparison of CPP-ACP, Tri-Calcium Phosphate and Hydroxyapatite on Remineralization of Artificial Caries Like Lesions on Primary Enamel -An *in vitro* Study

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***Objectives:*** *To compare CPP-ACP, Tri-calcium phosphate and Hydroxyapatite on remineralization of artificial caries like lesions on primary enamel.* ***Study design****: Ten extracted Primary molars coated with nail varnish, leaving a window of 2x4 mm on buccal and lingual surface were immersed in demineralizing solution for 96 hours and sectioned longitudinally to obtain 40 sections (4 sections per tooth) and were randomly divided into 4 groups (A to D) n=10; Group A: negative control, Group B: CPP-ACP, Group C: Tri-calcium phosphate, Group D: Hydroxyapatite. Sections were subjected to pH cycling for 10 days and were evaluated by polarized light microscope before and after treatment.* ***Results****: Intra group comparison of demineralization and remineralization was done by paired t-test. One way ANOVA was used for multiple group comparisons followed by post HOC TUKEY'S Test for group wise comparisons. Remineralization was found more with Group D followed by Group B, C and A.* ***Conclusion****: Hydroxyapatite showed better remineralization when compared to CPP-ACP and Tri-calcium phosphate.*

***Key words:*** *CPP-ACP, Demineralization, Hydroxyapatite, Polarized light microscopy, Remineralization, Tri-Calcium phosphate.*

## INTRODUCTION

Dental caries is a pathological process of localized destruction of tooth tissue by microorganisms. Over the last few decades, fluoride in various forms has been proven to reduce caries in both the primary and permanent dentitions when used in a variety of ways. [1] The enamel of primary teeth is more susceptible to caries development than that of permanent teeth due to lower mineral content and higher organic contents. [2] Crystals at the tooth surface regularly go through natural periods of mineral loss (demineralization) and mineral gain (remineralization).[3] The process of caries development is dynamic in which demineralization of the enamel is followed by remineralization which have a crucial impact on the hardness and strength of tooth enamel.[2]

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Various methods have analyzed tooth demineralization and remineralization, which includes both direct and indirect techniques. Several techniques have been used in Remineralization experiments like Scanning Electron Microscopy,[4] Microradiography,[5] Quantitative light-induced fluorescence,[6] Microhardness[7] and Polarized light microscopy.[8] The polarized light microscopy is a sensitive technique for assessing de- and remineralization in *in vivo* and *in vitro* studies.

CPP-ACP is derived from bovine milk protein, casein, calcium and phosphate, has been demonstrated to have anticariogenic potential in laboratory, animal, and human *in situ* experiments.[9,10] Tri-calcium phosphates has remineralization properties with the advantage of the calcium phosphate system, that is stable in aqueous environment and does not affect the fluoride activity when added to dentifrices.[11]

Hydroxyapatite is one of the most biocompatible and bioactive materials formed of nano-sized particles similar to the apatite crystals of tooth enamel morphology, crystal structure and crystalinity.[12] The aim of the present *in vitro* study evaluated and compared CPP-ACP, Tri-calcium Phosphate and Hydroxyapatite on remineralization potential of artificial caries like lesions on primary enamel.

## MATERIALS AND METHOD

Ten extracted non-carious primary molars were collected, thoroughly cleaned free of debris and calculus using hand scalers and stored in 10% formalin.

### De/Remineralizing solution preparation

The buffered remineralizing and demineralizing solutions were made from top-grade chemicals and deionized water. The demineralizing solution contained 2.2 mM $CaCl_2$, 2.2 mM $KH_2PO_4$, 0.05M acetic acid had the pH adjusted to 4.4 with 1 M KOH. The remineralizing solution contained 1.5 mM $CaCl_2$, 0.9 mM $NaH_2PO_4$, 0.15 M KCL had a pH of 7.0. This solution approximates to the super saturation of apatite minerals found in saliva.

The teeth were coated with an acid resistant nail varnish, leaving a narrow 'window', approximately 2x4 mm wide, on the intact surface on buccal or lingual surfaces. Each tooth was immersed in the demineralizing solution (10 ml) for 96 hours to produce artificial caries like lesions.

These demineralized teeth were mounted on an acrylic mold using self-cure acrylic resin with buccal and lingual half embedded in acrylic resin to obtain longitudinal sections through the lesion to produce four sections out of each tooth, approximately 150–200 μm thick using a hard tissue microtome. Forty enamel sections were made and stored in deionized water. Each section was mounted over the glass slide and examined for demineralized area at 40x magnification under a Polarized Light Microscopy (Olympus BX 51, Japan). The images were captured for each section and the depth of the lesion was measured using an image analyzer (Software Image Pro plus version 4.1.0.0 for Windows 95/NT/98,Media Cybernetics, USA). The sections showed a clear demarcation between sound enamel and the initial lesion. The depth of the lesion was determined at three different points from the outer surface to the deepest part of the demineralized area.

Forty sections (specimens) were randomly assigned to 4 groups (A to D) and were kept separately in deionized water -

**Group A:** sections with no treatment (negative control group)

**Group B:** sections were treated with CPP-ACP (GC tooth mousse, Recaldent TM, GC company, positive control group)

**Group C**: sections were treated with Tri-calcium Phosphate (Clinpro tooth crème, 0.21% sodium fluoride with fTCP, 3M ESPE company)

**Group D**: sections were treated with Hydroxyapatite (Remin-Pro, VOCO company)

Toothpaste and Tooth Mousse supernatant for Group B, C and D were prepared by suspending 15 g of the respective toothpaste/tooth mousse in 45 ml of deionized water, in order to achieve 1:3 (toothpaste: deionized water) ratio.

The sections were tied and tagged with 10 cm floss for ease of use and identification. These sections were then placed in the pH cycling system for 10 days. Total time for each cycle was 8 hrs /day. Each cycle involved three hours of demineralization twice a day with two hours of remineralization in between. Specimens in Group B, C and D were treated for 60 seconds with toothpaste supernatant (5 ml /section) both before and after the first and second demineralizing cycles. After the completion of each cycle per day, the sections were kept in deionized water, until required for use next day.

After the completion of pH cycle, sections were kept in acetone for 3 hours and then cleared in xylene and mounted on the glass slides with the DPX mounting medium.

### Evaluation technique (for measurement of the depth of the lesion)

After imbibition of the sections in water, these were examined at 40x magnification under polarized light microscopy to qualitatively evaluate the body of the lesions and the images were captured. Depth of the lesion was measured using an image analyzer (Software Image Pro plus) at three different points in each sample and values were compared before and after the experiment.

## RESULTS

The results were subjected to appropriate statistical analysis; mean ± standard deviation of lesion depth was calculated for each group. Among the three groups; group D (9.41 μm) showed the highest amount of remineralization compared to (4.61 μm) group A (Figure 1 and 2).

Intra group comparison of demineralization and remineralization was done by paired t-test. There was statistically highly significant value found (p-value of 0.001) following the remineralization in individual group (Table 1).

One way ANOVA was used for multiple group comparisons where Mean ± SD values were not significant (p-value 0.98) following demineralization but were highly significant following remineralization (p-value 0.001) (Table 2).

Post HOC TUKEY'S Test for group wise comparisons were not significant ($p>0.05$) after demineralization in each group. Following remineralization, there was a statistically significant difference found between Group A and B and highly significant difference between Group A and D (Table 3).

## DISCUSSION

The recent approach in caries management is the non invasive method. This method can transform a lesion from an active to an inactive state. Non-cavitated and cavitated lesions extending upto dentinoenamel junction can be arrested if the cariogenic challenge of certain microenvironment are sufficiently controlled and if therapeutic agents are applied for tissue healing. Professional delivery methods, such as toothpastes, gels, varnishes, fluoride releasing materials are commonly applied to remineralize high-risk areas. Complementing traditional diagnostic methods with advanced, more sensitive methods will improve caries diagnostic efficiency and hence the dental care and treatment of patients. [9,11]

In a primary tooth, enamel caries progress rapidly into the underlying dentin. For a carious process to proceed, the pH and the ionic activities of calcium and phosphate in plaque fluid are critical because they determine the stability of the tooth structure under cariogenic attack. Topical agents prevent and inhibit caries progression by inhibition of demineralization: and/or enhancement of remineralization, so creating a more caries resistant surface due to the remineralized crystals. [2, 13]

We investigated the changes in demineralization that indirectly reflected remineralization in advanced enamel lesions with similar depths as natural white spot lesions. Our

**Figure 1: Polarized microscope under 40X magnification, showing Demineralized area of enamel in Group D**

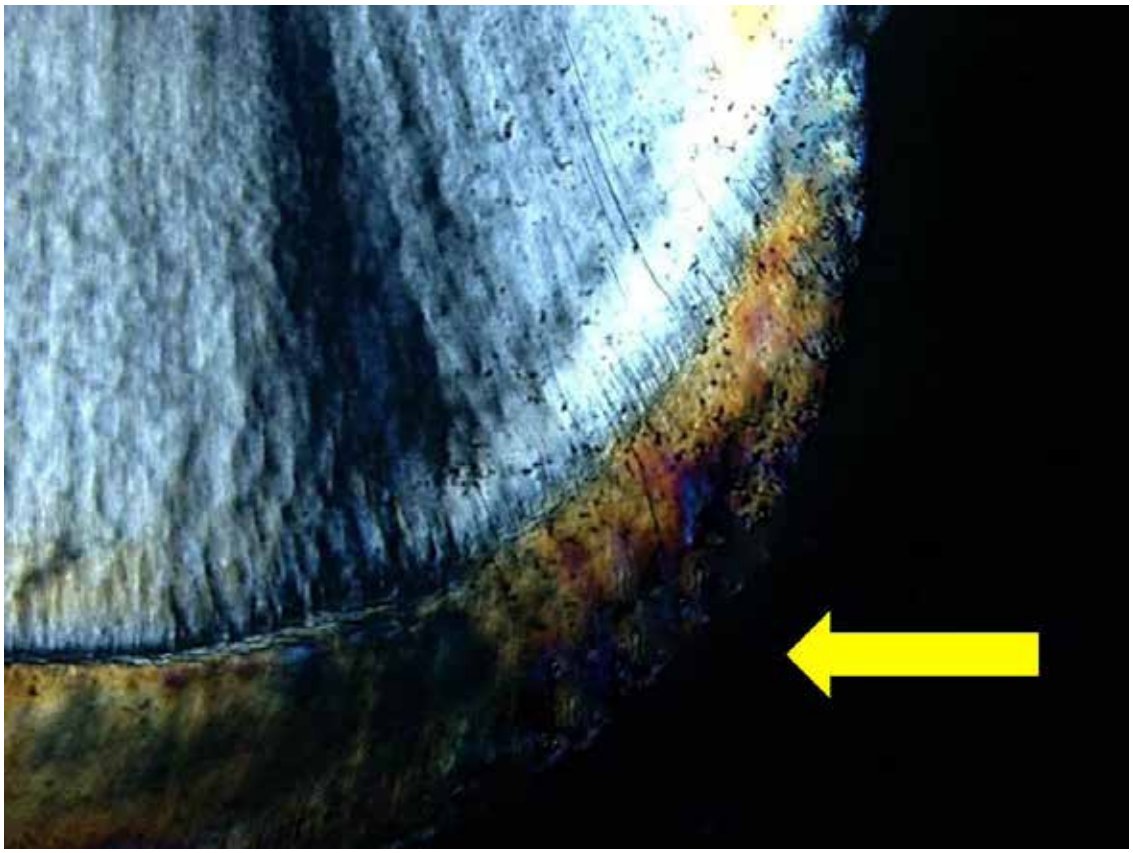
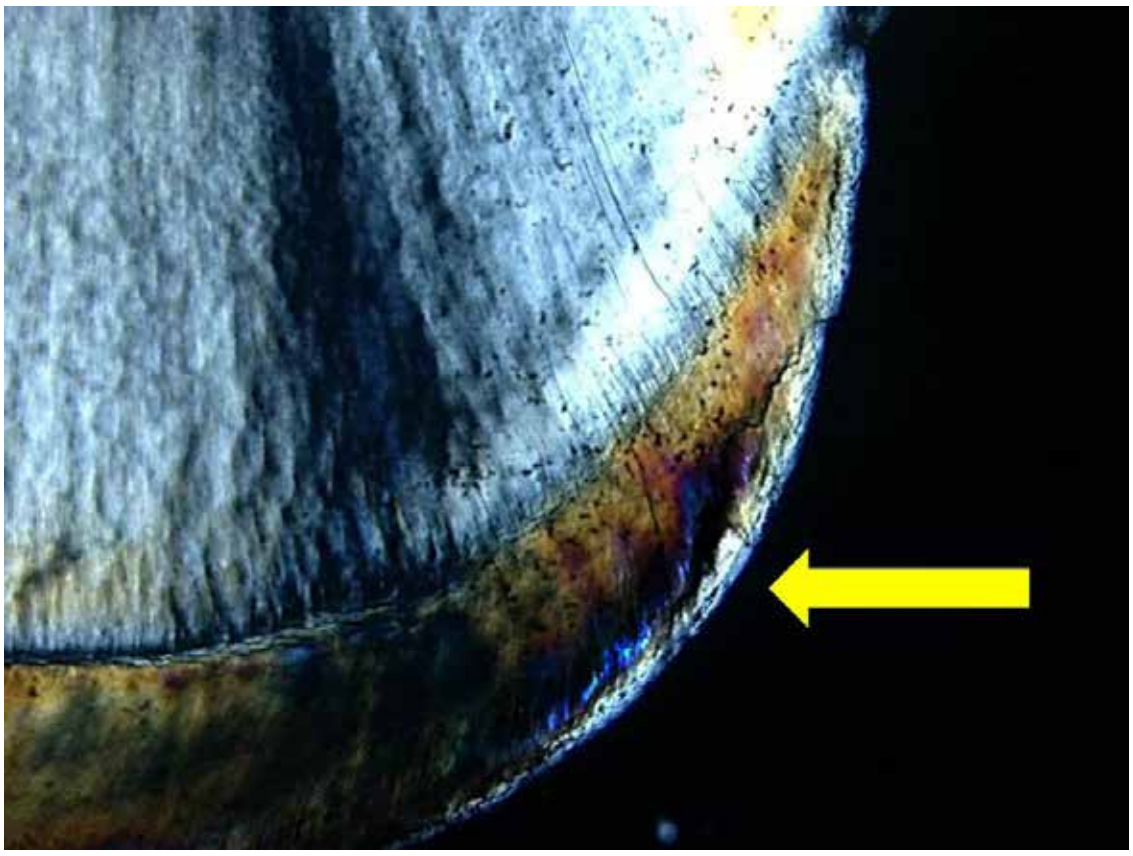


**Figure 2: Polarized microscope under 40X magnification, showing Remineralized area of enamel in Group D**



results showed that all agents had a significant reduction in the demineralized area and that differed significantly from each other. The remineralizing agents used in the present study were Hydroxyapatite (Remin pro), CPP-ACP (GC Tooth mousse) and Tricalcium phosphate (Clinpro tooth crème).

The tooth sections were subjected to a chemical caries model for the production of artificial caries lesion and pH cycling for testing the efficacy of remineralizing agents, which were sequentially exposed to demineralizing and remineralizing solutions with intermediary treatments with the agents. These methods have improved understanding of the mechanism of demineralization and remineralization. Further, they provide information about the effects of caries preventive agents on de/remineralization dynamics at the surface of the teeth.[15] Polarized light microscopy (PLM) analysis was chosen as it is extremely sensitive to changes in hard tissues. With respect to demineralization and remineralization, it can quantitatively show the areas of mineral loss and mineral gain represented by the visualization of areas with different porosities and birefringence.[14,16]

**Table 1: Mean lesion depth and Standard deviation values for all the four groups after Demineralization and Remineralization.**

| | Groups | Demineralization (µm) | | Remineralization (µm) | |
|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD |
| Group A | No treatment | 103.54 | 8.45 | 4.61 | 1.36 |
| Group B | CPP | 103.95 | 9.64 | 8.10 | 3.26 |
| Group C | TCP | 102.98 | 7.79 | 7.22 | 2.09 |
| Group D | HA | 104.35 | 7.78 | 9.41 | 2.95 |

*Student's paired t-test

**Table 2: Comparison of Mean ± SD values of depth of lesion in experimental and control groups.**

| | Groups | Demineralization (µm) | Remineralization (µm) |
|---|---|---|---|
| Group A | No treatment | 103.54 ± 8.45 | 4.61 ± 1.36 |
| Group B | CPP | 103.95 ± 9.64 | 8.10 ± 3.26 |
| Group C | TCP | 102.98 ± 7.79 | 7.22 ± 2.09 |
| Group D | HA | 104.35 ± 7.78 | 9.41 ± 2.95 |
| ANOVA | F | 0.05 | 6.45 |
| | P | 0.98, NS | 0.001**, HS |

One way ANOVA test

**p< 0.001=HS Highly significant

**Table 3: Comparison of difference between groups**

| Groups | P value of difference in Demineralization | P value of difference in Remineralization |
|---|---|---|
| A-B | NS | 0.02*, S |
| A-C | NS | 0.11, NS |
| A-D | NS | 0.001**, HS |
| B-C | NS | 0.86, NS |
| B-D | NS | 0.66, NS |
| C-D | NS | 0.23, NS |

Post-hoc Tukey's Test
*P < 0.05, S
**P < 0.001, HS
P > 0.05, NS

The early enamel caries could be divided into four zones based on its histological appearance under polarized light microscope, i.e. - translucent zone, body of lesion, dark zone and surface zone. There is a translucent zone at the inner advancing front of the lesion, while a dark zone may be found superficial to this. The body of the lesion which is the major part is the third zone lying between the dark zone and the surface enamel. The surface zone lies above on the outer side. Dark zone and surface zone are the positively birefringent zones and showed signs of remineralization which was similar in a study done by Bansal *et al* 2010.[17] The process of remineralization was observed starting from the outer surface of the lesion towards inner surface.

CPP-ACP is the acronym for a complex of casein phosphopeptides (CPPs) and amorphous calcium phosphate (ACP). It has been proposed that the anticariogenic mechanism of CPP-ACP is due to localization of ACP at the tooth surface which then buffers the free calcium and phosphate ion activities, thereby helping to maintain a state of supersaturation with respect to enamel, so depressing demineralization and promoting remineralization.[18]

The remineralizing potential of CPP-ACP has been shown in animal studies,[18] *in vitro* [9,19,20] and *in vivo* studies.[10,21] Several papers have also shown that higher concentration of CPP-ACP elicit higher remineralization.[10] The concentration of CPP-ACP used in the trials varied from 2% to 10% w/w. It has been reported that CPP-ACP produce a similar remineralization effect as that of 2800 ppm F at 2% w/w concentration,[22] and could efficiently promote enamel remineralization at 3% w/w.[23] CPP-ACP used for the present study was 10% w/w in concentration We found an increased enhancement of remineralization by CPP-ACP when compared to Tricalcium phosphate with 950 ppm F, whereas decreased enhancement seen when compared to hydroxyapatite with 1450 ppm F.

In a previous *in vitro* study, CPP-ACP when used in combination with fluorides showed better results and lower caries score than when used individually.[1,24] This was probably due to the ability of CPP-ACP to interact with fluoride ions to produce an additive anticariogenic effect through the formation of a stabilized amorphous calcium fluoride phosphate phase. Conversely, research also suggests that CPP-ACP cream is not as effective as fluoride in remineralizing early enamel caries at surface level. Combination of fluoride and CPP-ACP does not provide any additive remineralization potential compared to fluoride alone. [25]

Rehder Neto *et al* in 2009 compared the remineralization lesion potential of CPP-ACP and CSP (calcium sodium phosphosilicate) containing paste on acid softened enamel. They compared 4 products with control, (i) CPP-ACP (MI paste) (ii) CPP-ACP + Fluoride (MI paste plus) (iii) CSP (tooth revitalizing paste) (iv) Fluoridated dentrifices (FD Sensodyne cool gel) (v) control, and concluded that, the increase in surface microhardness in CPP-ACP group did not differ significantly and was higher than the control group.[26] However, in the present study, although CPP-ACP was used alone without the combination of fluoride, it showed significant increase in remineralization and decrease in lesion depth when compared to tricalcium phosphate containing fluoride. The advantage of using CPP-ACP as a supplement to fluoride-containing products is still unclear. High-quality, well designed clinical studies in this area are still required before definitive recommendations are made.[27]

Tricalcium phosphate (TCP) has been considered as one possible means for enhancing the levels of calcium in plaque and saliva. Combining calcium phosphate and fluoride ions in oral care products is problematic and can lead to loss of bioavailable fluoride ion due to a reaction between the calcium phosphate phase and the fluoride ion. In an approach to overcome this incompatibility of calcium phosphates and fluoride ions, new technologies have been developed.[22,28] This technology supports functional tricalcium phosphate (fTCP) where tricalcium phosphate particles have been ball milled with sodium lauryl sulfate, and has been included in a tooth crème with sodium fluoride marketed as Clinpro tooth crème (3 M ESPE).[28]

In a previous *in vitro* study, NaF (5000 ppm) showed the highest degree of remineralization when observed from GC MI paste plus (CPP-ACP + 900 ppm of fluoride) and Clinpro tooth crème (TCP + 950ppm of fluoride) which were found to be comparable.[14, 29] This observed response showed that F-deposition during treatment depends on lesion depth. With elevated external F-levels, the F-gradient will be higher, driving the fluoride deeper into the lesion, in spite of the F-diffusion being slowed by adsorption onto and reaction with hydroxyapatite crystallites in the pore walls. [30]

Vanichvatana *et al* tested the efficacy of two calcium phosphate pastes (Tooth mousse plus with 900 ppm of fluoride and Clinpro tooth crème with 950ppm of fluoride) fluoride toothpaste (Colgate Palmolive with 1000ppm of fluoride) on remineralizing artificial caries using polarized light microscopy and concluded that Clinpro tooth cream showed similar benefits compared to fluoride toothpaste and had no additional benefits of tooth mousse plus.[31] However, Clinpro tooth crème in the present study showed least amount of remineralization effect when compared to CPP-ACP and Hydroxyapatite.

Hydroxyapatite (HA) is one of the most biocompatible and bioactive materials and is widely applied to coat artificial joints and tooth roots.[24] Nano-sized particles have similarity to the apatite crystal of tooth enamel in morphology, crystal structure and crystallinity.[32] In the present study hydroxyapatite (Remin pro) was used as remineralizing agent, which is a water- based cream, containing hydroxyapatite, fluoride and Xylitol. Hydroxyapatite which is the main constituent of Remin Pro fills the superficial enamel lesions and the tiniest irregularities that arise from erosion. Fluoride, which is also one of the content of Remin pro gets converted to fluorapatite when it comes in contact with saliva; thus strengthens the tooth and renders it more resistant to acid attacks.[33] Since the surface area and proportion of the atomicity increase with decreasing particle size, nano-HA has bioactive and biocompatible properties.[34]

Uday *et.al* 2013 assessed the effect of Remin pro on bleached enamel and concluded that Remin pro causes an increase in the microhardness. The author believes this is due to the presence of 1450 ppm fluoride, which is 61% higher than other available agents today.[33] In the present study hydroxyapatite (Remin pro) showed significant result in surface remineralization when compared with CPP-ACP and tricalcium phosphate.

One must bear in mind that remineralization *in vitro* may be quite different when compared with dynamic, complex biological system, which occurs naturally in the oral cavity. Thus, direct extrapolations to clinical conditions must then be exercised with caution However, there is a need for further long term research under clinical conditions to prove the efficacy of these agents.

## CONCLUSION

All the three groups viz. CPP-ACP, TCP and HA showed remineralization under *in vitro* pH cycling model.

HA group showed significantly more remineralization compared to CPP-ACP and TCP.

Remineralization was observed from the surface towards the lesion.

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