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Remineralization of Incipient Lesions with Anticariogenic Casein Phosphopeptides and Fluoride: A Vivo Study

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Abstract

Objective: The aim of this study was to evaluate the remineralization of incipient lesions with CPP-ACP and its comparison with the effect of fluoride on the same. **Study Design:** A total of 225 teeth with incipient lesions in 108 children within the age group of 6-14 yrs. were divided into five groups by block randomization. Enamel biopsy was done for incipient lesions. Calcium and phosphate were analyzed using auto analyzer, fluoride was assessed using Orion analyzer. **Results:** Results showed that CPP-ACP enhances the remineralization process and that CPP-ACP has comparable effect as fluoridated dentifrice, as difference was not statistically significant ($p>0.05$), using ANOVA, Tukey's test. **Conclusion:** CPP-ACP is effective remineralizing agent. A beneficial dose response effect is seen with the application of casein phosphopeptides. In addition, there is a synergistic effect of CPP-ACP with fluoride.

Keywords: CPP-ACP, Fluoride, Remineralization

1. Introduction

Clinically detectable white spot lesions could "involute" or remineralize over time. Head in 1909[1] first described the phenomenon of re-mineralization. There is ample opportunity to facilitate natural re-mineralization which would otherwise require up to 6-7 years.

Fluorides enhance remineralization[2]. Recently, a number of bioactive milk-based components have been identified as having a potential role in prevention of dental caries. Casein Phosphopeptides (CPP) are a group of peptides derived from casein, part of the protein in milk. The CPP renders calcium and phosphate soluble. In this, amorphous state calcium and phosphate ions are able to penetrate tooth enamel. Thus, reducing enamel demineralization and promoting remineralization[3].

Also, CPP-ACP is known to have a synergistic effect with fluorides. Fluoride has the capacity to improve the crystalline tooth structure that would assist the improvement of acid resistance of CPP-ACP[4].

The present study evaluates the remineralization of incipient lesions with CPP-ACP and its comparison with the effect of fluoride on the same.

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2. Materials and Methods

The present study was conducted in the Department of Pedodontics and Preventive Dentistry, Manipal College of Dental Sciences, Manipal and Ethical clearance for this study was obtained from the Institutional Review Board of Manipal College of dental science, Manipal.

The Sample size consisted of a total of 225 teeth with incipient lesions in 108 children, within the age group of 6-14 years. This study was carried out in two residential schools in Udupi district. Before the commencement of the study, an informed consent from the Principal of the school as well as the parents of the students participating in the study was obtained. Selection criteria were based on similar socioeconomic status, dietary habits, oral hygiene methods, oral hygiene status and children who had incipient lesions.

The materials used in the study were Filter paper, 0.5 mol/litre perchloric acid, double distilled water for carrying out enamel biopsy; Recaldent-GC Mousse containing CPP-ACP for application of CPP-ACP, along with regular diagnostic and scaling instruments. Orion ion analyzer was required for fluoride estimation, while Auto analyzer was used for calcium and phosphate analysis.

The group distribution of 225 teeth with incipient lesions was done by block randomization into five groups having 45 teeth with incipient lesion in each-

Group I - Nonfluoridated toothpaste used twice daily. (negative control)

Group II - Fluoridated toothpaste used twice daily (positive control).

Group III - Weekly professional application of CPP-ACP for one month, with Nonfluoridated toothpaste.

Group IV - Daily self and weekly professional application of CPP-ACP for one month, with Nonfluoridated toothpaste.

Group V - Weekly professional application of CPP-ACP for one month, with fluoridated toothpaste.

An initial recording of baseline gingivitis, plaque indices and caries (incipient lesion) scores were done. Oral prophylaxis using ultrasonic scaler was carried out for all subjects in order, to obtain gingivitis and plaque scores to zero. Enamel biopsy of the incipient lesions was performed to assess calcium, phosphate and fluoride content. In groups III, IV and V professional application of CPP-ACP was done. All groups were instructed on oral hygiene measures, their usage and were kept under supervised brushing schedule.

3. Procedure for Enamel Biopsy

Surface enamel biopsies were taken in-vivo ***(Vogle)***[5]. Briefly, the surface of the tooth was cleaned, washed and dried. Isolation was done with the help of rubber dam to eliminate any chances of salivary contamination. A 4mm diameter non-fluoride containing filter paper circle was wetted with 10 micro liters of 0.5 mol/liter perchloric acid and immediately placed on the mesiobuccal surface of the tooth and was timed for 15 seconds. This filter paper was then transferred to a container which had 20 ml of double distilled water pipetted using a glass syringe.

4. Application of CPP-ACP

Excess saliva on the tooth surface was removed. CPP-ACP in the tray was applied to the upper and/or lower teeth and left undisturbed for 3 minutes. After the removal of the tray the patient was instructed to use the tongue to spread the remaining CPP-ACP throughout the mouth and to retain for as long as possible (1-2 minutes), avoiding expectoration and delaying swallowing. Also, the patient was advised not to eat or drink for 30 minutes following application.

Similar, procedure was repeated for children in group III, IV and V. Professional application was done at regular weekly intervals for one-month period.

Estimation of calcium and phosphate was done using Roche Hitachi 901 Autoanalyzer. 10 microliters of the sample were taken into autoanalyzer test tubes and commercially available reagents were added to the samples.

***For calcium*:** the first reagent R1 (buffer) was added to biopsy sample followed by addition of R2 (chromogen) to initiate the reaction. The intensity of purple color complex formed was directly proportional to calcium concentration which was measured photometrically, based on working principle of Colorimetric assay with an endpoint determination.

***For phosphates*:** In the biopsy sample R1 reagent was added followed by addition of R2 (phosphate reagent) to initiate the reaction. The complex formed was determined photometrically in the ultraviolet region (340nm), based on the working principle of end point method with sample blanking.

Estimation of fluoride (Frant and Ross)[6] 10 ml TISAB (total ionic strength adjustment buffer) was added using a plastic dispenser to the plastic container, after which the plastic container with the biopsy sample was stored for three days to obtain maximum fluoride diffusion into the diluent of double distilled water and TISAB.

After three days of storage the sample was stirred with a magnetic stirrer. The Orion Ion Analyzer 901 was standardized using standard fluoride solutions. Three standard solutions were prepared and after adjusting the voltage (mV), the known concentrations of fluoride standards were analyzed. Following standardization of the Orion Ion Analyzer, each biopsy sample was subjected to the specific fluoride electrode for a minimum of 5 minutes or till the reading on the analyzer was stabilized for a minimum period of 3 minutes. After the stabilization, the reading (fluoride level in ppm) was noted.

5. Follow -Up

Subsequent follow up was done at one-month interval. The plaque and gingivitis scores, mineral content using enamel biopsy was recorded.

6. Statistical Analysis

Intra-group comparison was done using the paired 't- test'; while for the inter-group comparison ANOVA followed by Tukey's test was applied. All the tests were carried out using the computer based SPSS package.

7. Results

A total of 225 teeth with incipient lesions in 108 children within the age group of 6-14 years were divided into five groups by block randomization. Each group had 45 teeth with incipient lesions. The overall attrition of the entire sample was 7.11%, which is within acceptable limits.

7.1 Intra-group comparison of calcium, phosphate and fluoride scores after active intervention

The non-fluoridated toothpaste group (i.e. group I) showed no significant difference (p>.05) when compared to baseline. For group II using fluoridated toothpaste highly significant increase (p<.01) in mean calcium and fluoride scores, with a significant increase in phosphate scores (p<.05) at the end of one month was noted. In group III, where CPP-ACP was professionally applied, with regular use of non-fluoridated toothpaste, highly significant increase in calcium, phosphate scores was found (p<.01), using paired sample t-test.

A highly significant (p<.01) increase in calcium and phosphate scores was shown by Group IV. For Group V in whom, along with professional application of CPP-ACP, fluoridated toothpaste was used, highly significant increase in scores of calcium and phosphate, with a significant increase in fluoride was seen.

7.2 Inter-group comparison of mean calcium, phosphate and fluoride scores after active treatment (Table 1 A, B; 2 A, B; 3 A, B)

On application of ANOVA test of significance highly significant difference (p<.01), indicated that treatment groups were comparable. Tukey's test was then applied for inter-group comparison of calcium, phosphate and fluoride scores after one month of active treatment.

Calcium scores

The result obtained suggests that group II, IV and V had significantly greater rise in calcium as compared to group I and III. Comparison of group II, III, IV to group V showed group V to be highly significant(p<.01).

Phosphate scores

Tukey's Test showed a significant difference was observed when groups III and IV were compared to group I, (p<.05). On comparison of group III (professional application) to group IV significant difference was observed (p<.05). Group V showed highly significant increase in phosphate values in comparison to Group I, II, III and IV (p<.01).

Table 1. **A,B:** Intergroup calcium one month after active intervention

Groups	Mean Calcium ± SD	p-value
Group I	0.748 ± 0.35	0.001*
Group II	1.047 ± 0.29	
Group III	0.968 ± 0.42	
Group IV	1.137 ± 0.29	
Group V	2.025 ± 0.85	

ANOVA test 'Á'

Groups	p value
Group I vs. Group II	0.022*
Group I vs. Group III	0.160
Group I vs. Group IV	0.001*
Group I vs. Group V	0.000*
Group II vs. Group III	0.933
Group II vs. Group IV	0.894
Group II vs. Group V	0.000*
Group III vs. Group IV	0.010*
Group III vs. Group V	0.000*
Group IV vs. Group V	0.000*

Tukey test 'B'

Where * indicates groups significantly different at p<0.05.

Table 2. **A,B:** Intergroup phosphate score one month after active intervention

Groups	Mean Phosphate ± SD	p-value
Group I	0.171 ± 0.13	0.001*
Group II	0.247 ± 0.17	
Group III	0.293 ± 0.19	
Group IV	0.217 ± 0.12	
Group V	0.393 ± 0.26	

ANOVA test 'A'

Groups	p value
Group I vs. Group II	0.272
Group I vs. Group III	0.011*
Group I vs. Group IV	0.032*
Group I vs. Group V	0.000*
Group II vs. Group III	0.759
Group II vs. Group IV	0.938
Group II vs. Group V	0.005*
Group III vs. Group IV	0.035*
Group III vs. Group V	0.001*
Group IV vs. Group V	0.002*

Tukey test 'B'

Where * indicates groups significantly different at p<0.05.

Table 3. **A,B:** Intergroup fluoride score one month after active intervention

Groups	Mean Fluoride ± SD	p-value
Group I	0.079 ± 0.01	0.001*
Group II	0.090 ± 0.01	
Group III	0.086 ± 0.14	
Group IV	0.084 ± 0.16	
Group V	0.119 ± 0.72	

ANOVA test 'A'

Groups	p value
Group I vs. Group II	0.048*
Group I vs. Group III	0.893
Group I vs. Group IV	0.962
Group 1 vs. Group V	0.001*
Group II vs. Group III	0.971
Group II vs. Group IV	0.912
Group II vs. Group V	0.002*
Group III vs. Group IV	0.999
Group III vs. Group V	0.001*
Group IV vs. Group V	0.002*

Tukey test 'B'

Where * indicates groups significantly different at p<0.05

Fluoride scores

After one month of active treatment, group I was found to be significantly different from group II (p<.05). Highly significant increase in fluoride values was seen in group V when compared with group I, II, III and IV, (p<.01).

Plaque and Gingivitis scores

No significant difference was seen on Inter-group comparison of mean plaque scores after 1 month of active treatment within all groups. Inter-group comparison of mean gingivitis scores of group I to group II, III and IV showed no significant results [p>0.05]. However, comparison of group I and III to Group V showed highly significant difference(p<.01).

8. Discussion

Residential schools were chosen such that subjects could be kept under supervised brushing and diet control. Group I served as the control group and was included in the study to rule out the effect of the mechanical cleansing. Fluoride dentifrices were chosen as Robert[7] reported that CPP-ACP produced a reduction in caries activity similar to that of a 500 ppm fluoride solution.

Group V showed a significant attrition of 28.88% but the overall attrition of the sample (7.11%) was within acceptable limit. This attrition was because the students, who changed schools, were probably more in group V.

The remineralizing and anticariogenic efficacy of the casein phosphopeptides (CPP-ACP) was tested against the increase in mineral contents (calcium, phosphate and fluoride level) of the tooth; Plaque score by Silness and Loe[8], Gingivitis score by Loe and Silness[9]. The idea behind assessment of the mineral content was to know the quantitative changes in mineral content of the incipient caries lesion during remineralization with or without addition of an accelerator (i.e. fluoride/CPP-ACP/both).

8.1 Calcium, Phosphate, Fluoride Scores After - Active Intervention

There was no significant increase in the calcium, phosphate and fluoride scores from baseline to the end of one month in group I, this finding can be attributed to the slow natural process of remineralization due to supervised mechanical removal of plaque. Similar findings have been reported in a study conducted by Creanor *et al.*[10].

Fluoride dentifrice was effective in enhancing remineralization process, as can be conclude from the increase in calcium, phosphate and fluoride scores in group II. Similarly, the observation made by Joyston and Kid[11], Ashirov[12] in their study confirmed the contribution of fluoride contained in the toothpaste as being the major

component of enamel remineralization at the stage of its maturation in young children.

Highly significant increase was seen in calcium and phosphate scores, with no significant difference in the fluoride score in group III (only professional application group) and IV (daily self-application group) subjects, using non-fluoridated toothpaste for one month. These findings suggest that CPP-ACP is effective in enhancing the process of remineralization and that mineralization was mainly in form of hydroxyapatite. Effectiveness of CPP-ACP in the process of remineralization was also shown by Shen *et al.*[13], Iijima *et al.*[14].

In group V, subjects were applied CPP-ACP at weekly intervals professionally along with use of fluoridated toothpaste. There was highly significant increase in calcium, phosphate and fluoride scores at the end of one month implying a synergistic effect of CPP-ACP with fluoride. This is consistent with work of Blake *et al.*[15] who studied the effect of a two-step treatment (consisting of a calcium rinse followed by a fluoride treatment) and concluded that a two step treatment produced a 100% increase in calcium content of model plaque, presumably due to the formation of CaF_2, compared with treatment done with artificial saliva followed by calcium alone.

In a study conducted by Schemehorn *et al.*[16] it was demonstrated that calcium and phosphate supplementation in toothpaste or mouth rinse can improve remineralization and increase fluoride uptake.

8.2 Inter-Group Comparison after Active Intervention

Non-fluoridated dentifrices *vs*. fluoridated dentifrices

An inter-group comparison between the groups I and II revealed that there was significant increase in calcium and fluoride scores in group II; phosphate scores between the two groups showed no significant difference. This difference observed between calcium and phosphate score can probably be due to the difference in the sensitivity of these two mineral contents. Phosphates require more concentration to have significant effect on remineralization in presence of fluoride. This finding can be supported by a study conducted by Tanaka and Kodama[17] who found that phosphates needed 20 times more concentration than calcium to impart inhibitory effect on enamel demineralization compared to calcium.

The results showed that fluoride-containing toothpaste produced a significant increase in mineral content of calcium and fluoride as compared to the non-fluoridated toothpaste.

8.3 Fluoride verses CPP-ACP

On comparing group II with group III and IV no significant difference was observed between calcium scores ($p>0.05$), phosphate scores ($p>0.05$) and fluoride scores ($p>0.05$). The results suggest that CPP-ACP was comparable to fluoride dentifrices in enhancing the process of remineralization. Similarly, the use of a crude CPP preparation at 5.0% w/w in a dentifrice was reported by Robert[7] to significantly inhibit enamel demineralization in a human intra-oral caries model. The inhibition obtained in their study was similar to that obtained with a 500 ppm fluoride dentifrice.

Comparison of the caries preventive efficacy of a mouth rinse solution containing casein derivatives coupled with calcium phosphate (CD-CP) by Hay and Morton[18] with that of a 0.05% sodium fluoride mouthrinse among individuals (124) having dry mouth, also corroborates to the present findings. Coronal caries incidence was higher in the sodium fluoride group than in the CD-CP group (34.4% and 27%, respectively), but the difference was not statistically significant.

8.4 Effect of CPP-ACP with Fluoride (Group V)

The result of the combined application group was superior implying the synergistic action of CPP-ACP and fluoride.

Inter-group comparison of group II to V; group III to V and group IV to V resulted in the highly significant increase in calcium, phosphate and fluoride scores in group V, thus implying that combined application of CPP-ACP with fluoride has better effect in enhancing the remineralization potential of fluoride, or vice-versa.

The observations of the present study is in agreement with the fact stated by Varughese and Moreno[19] that fluoride is most effective in inhibiting HAP dissolution when calcium and phosphate ions are also present in solution (plaque/pellicle/saliva). Similar findings were reported by Koulourides[20], Racquel *et al.*[21], Zhao and Cai[22].

The synergistic effect could be explained as fluoride decreases the affinity of bacterial calcium binding. Introduction of fluoride into the system decreases the binding affinity thereby increasing the rate of calcium

effusion by 20 to 48 %. Thus, fluoride significantly increases calcium mobility in plaque; this may increase the rate at which calcium is transported between plaque and an underlying lesion and so promote remineralization, as shown in the study of Rose and Turner[23]. Also, fluoride has the capacity to improve the crystalline tooth structure, thereby the acid resistance of CPP-ACP containing material gets improved by the addition of fluoride as in report by Kariya *et al.*[4]. Further it has been suggested by Reynolds *et al.* that fluoride inhibit the phosphatase and peptidase enzyme produced by dental plaque bacteria which can destroy the ionic binding properties of CPP. Thus, enhancing the effect of CPP-ACP.

8.5 Plaque and Gingivitis Score after Active Intervention

The decline in plaque scores could be attributed to the supervised brushing schedule. Rolla and Bjorn[24] stated improved oral hygiene and that the use of fluoride may be beneficial; the two procedures having synergistic effect.

There was significant difference in the gingivitis scores on comparing group I to group V and group III to group V. An additive effect of fluoride and CPP-ACP on gingival health was seen. This could be explained by the fact that, while CPP-ACP mainly affects the adherence of microorganisms[25], fluorides anti-microbial effect is intracellular[26]. When both CPP-ACP and fluoride is present there may be an additive effect.

Observations of the present study indicate a beneficial dose response effect of CPP-ACP on remineralization. CPP-ACP is comparable to fluorides in enhancing the naturally occurring process of remineralization. There exists a synergistic action of CPP-ACP with fluoride. But the use of CPP-ACP has to be with caution in children allergic to milk protein.

9. Conclusion

The results of this study show that,

1. CPP-ACP effectively enhances the process of remineralization.
2. A beneficial dose response effect is seen with application of casein phosphopeptide.
3. Mineralization with CPP –ACP is mainly in form of hydroxyapattite, as shown by significant increase in only calcium and phosphate scores.
4. CPP-ACP appears to be comparable to fluoridated dentifrice.
5. There is a synergistic effect of CPP-ACP with fluoride on mineralization with an additional effect on gingival health.

Therefore, CPP-ACP may be used as equally effective preventive tool as fluoride against acid attack. CPP-ACP is a natural derivative of milk therefore unlike fluoride they could be added to sugar-containing foods and confectionery without adverse organoleptic effects. Also, CPP-ACP has a potential as a toothpaste additive to improve the efficacy of the current fluoride-containing dentifrices. Thus, CPP-ACP would also have an important role in toothpastes either alone or with low fluoride for children at risk of fluorosis.

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