Original Article

Comparative Evaluation of the Efficacy of Tricalcium Phosphate, Calcium Sodium Phosphosilicate, and Casein Phosphopeptide – Amorphous Calcium Phosphate in Reducing *Streptococcus mutans* Levels in Saliva

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ABSTRACT

Background: There are only limited studies that have determined the antibacterial effects of various remineralizing agents that can be beneficial to children. **Aim:** The aim of this study is to compare the efficacy of tricalcium phosphate (TCP), calcium sodium phosphosilicate (CSP), and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) enhanced with fluoride in reducing the *Streptococcus mutans* (SM) levels in saliva of children. **Materials and Methods:** Out of 245 children, 120 of them with SM colony forming units (CFU)/ml in the range of 10^4–10^6/ml of saliva were assigned to four groups: (I) TCP; (II) CSP; (III) CPP-ACP enhanced with fluoride; and (IV) control. Salivary samples were collected at intervals of 1 week, 2 weeks, and 4 weeks and the number of CFU/ml of SM in saliva were counted post 48 hour incubation. **Results:** After 1 week, 2 weeks, and 4 weeks, there was a significant reduction in the mean score of SM ($P < 0.05$). The maximum reduction in the CFU/ml in the saliva was seen in the 1st week after the commencement of the brushing in all the three test groups. Group III children demonstrated the maximum reduction of 15×10^5 CFU/ml, followed by Group II children with 10×10^5 CFU/ml. **Conclusions:** Twice daily use of CPP-ACP with fluoride, CSP, and TCP caused a significant reduction in the levels of SM in saliva.

KEYWORDS: *Calcium sodium phosphosilicate, fluoride, saliva, Streptococcus mutans, tricalcium phosphate*

INTRODUCTION

Streptococcus mutans (SM) possesses a unique array of putative cariogenic trait[1] and a very high hierarchical status among the plaque microbiota with respect to their acid tolerance and their acidogenicity in an acidic plaque milieu.[2,3] Concentrations of SM in dental plaque regularly exceeding 50% of the total cultivable flora or averaging about 10% of the salivary flora have been reported.[1,4] Collectively, the existing data indicate that it is reasonable to identify caries-susceptible individuals from the correlation between the presence of SM and the development of dental caries.[5,6] When the carious lesion progresses, acid produced by bacterial action on dietary fermentable carbohydrates diffuses into the tooth and dissolves the carbonated hydroxyapatite mineral by demineralization.[7] It has been proved that demineralized enamel could be remineralized if there is no mechanical deformation of the enamel or breach in the surface layer.[8] The demineralization and remineralization are dynamic processes in the initiation and progression of caries lesions.[9] It has been accepted that remineralization is a noninvasive approach for restoring carious teeth, at least during the earlier stages of the caries process.[10]

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With the understanding of the dynamics of dental caries, several materials have been developed that have the potential to remineralize the tooth structure. It has been widely accepted that fluoride has a profound effect on caries prevalence by preventing and arresting caries, but it is far from a complete cure. Materials such as tricalcium phosphate (TCP), calcium sodium phosphosilicate (CSP) (bioactive glass), and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) enhanced with fluoride possess the ability to remineralize the tooth as these contain mineral ions which are similar to those present in enamel and dentin. CPP contains a cluster of phosphoseryl residues in the motif Ser(P)-Ser(P)-Ser(P)-Glu-Glu which markedly increase the apparent solubility of calcium phosphate[11] by stabilizing ACP under neutral and alkaline conditions forming metastable solutions that are supersaturated with respect to the calcium and phosphates.[12] The synergistic effect of CPP-ACP and fluoride in reducing caries experience may be attributable to the formation of CPP-stabilized amorphous calcium fluoride phosphate resulting in the increased incorporation of fluoride ions into plaque together with increased concentrations of bioavailable calcium and phosphate ions.[13]

CSP (bioactive glass) has numerous novel features, most important of which are its ability to act as a biomimetic mineralizer, matching the body's own mineralizing traits, while also affecting cell signals in a way that benefits the restoration of tissue structure and function.[7] This is attributed to the fact that the current standard treatment for tooth remineralization and prevention of decay is slow acting and is dependent on adequate saliva as a source of calcium and phosphorus.[14] TCP is a source of calcium and phosphate that are seen in many dental preparations such as toothpastes and rinses. TCP is not only one specific molecule but also a collection of calcium oxides, calcium phosphates, and free phosphate groups. The dissolution of TCP at oral pH levels releases only moderately greater amounts of calcium and phosphate ions than dicalcium phosphate.[15]

CPP-stabilized ACP acts as a salivary biomimetic that provides calcium and phosphate ions in a bioavailable form allowing these ions to inhibit demineralization and promote the remineralization of early caries lesions.[16,17] Chandak *et al*.[18] found a reduction in scores of *S. mutans* count after application of fluoride varnish and CPP-ACP in children. It was concluded that fluoride varnish, CPP-ACP, and CPP-ACP plus fluoride protect the tooth structure, preserving the integrity of primary dentition, with the most encouraging results being with CPP-ACP plus fluoride. An *in vitro* study on the effect of aqueous CPP-ACP on *S. mutans* biofilm significantly altered and reduced the biofilm development compared to controls. It also disrupted established *S. mutans* biofilms, and the study concluded that the use of CPP-ACP-containing glass ionomer cements (GICs) combined with regular CPP-ACP treatment may lower *S. mutans* challenge.[19] However, a study by Pinheiro *et al*.[20] showed no additional antimicrobial properties to GIC benefit of adding CPP-ACP. A clinical study among dental students showed that the daily consumption of chewing gum containing CPP-ACP significantly reduced the level of salivary *S. mutans than* chewing gum containing xylitol.[21]

Most of the studies relating to these materials have focused mainly on the remineralizing potential.[22] These materials have proven to be competent remineralizers. There are only limited studies in the literature that have determined the antibacterial effects of various remineralizing agents that can be beneficial to children. Hence, this present study was carried out to evaluate and compare the efficacy of TCP, CSP, and CPP-ACP with fluoride in reducing the SM level in stimulated whole saliva samples obtained from children.

Materials and Methods

Ethical approval

The present study was carried out in the Department of Pediatric and Preventive Dentistry in collaboration with the Department of Medical Microbiology, Saveetha University, Chennai, India. The study protocol was developed and ethical clearance was obtained from the Ethical Committee, Saveetha University, Chennai, India. Permission was sought from the school authorities of the Aacini Matriculation Higher Secondary School, Ambattur, Chennai. The purpose of the study and the protocol involved was explained to the parents of the participating children in the language they best understood. An informed written consent was obtained from the parents of all the children before their participation in the study.

Sampling and selection criteria

Five hundred children between the age group of 3–8 years were initially selected for the study. The inclusion criteria were medically healthy and caries-free children with a primary dentition or mixed dentition, children with no history of antibiotic use over 3–4 weeks before the study, and those with no history of fluoride treatment over 2 weeks before the study. Children not willing to participate and those with tooth structure defects were excluded from the study. Two hundred and forty-five children who

fulfilled these above mentioned criteria were selected for the study.

Saliva collection

The selected children were asked to refrain from eating or drinking (except water) 1–2 h before collection of the salivary samples. Each child was asked to chew on a block of paraffin and expectorate for a minute into a sterile plastic container. Two milliliters of stimulated whole saliva samples were thus collected and individually coded. These salivary samples were hermetically sealed and transported in a case containing ice.

Quantitative analysis

The microbiological tests based on the previous research[23] were carried out within 60 min after collection. Saliva samples were identified by the code number assigned. Each saliva sample was diluted 1:100 in sterile phosphate-buffered saline by means of a micropipette (0.1 mL aliquot of saliva was aseptically vortexed with 4.9 ml of sterile phosphate-buffered solution). Using an inoculation loop (4 mm in diameter), 0.1 ml of vortexed sample was streaked on mitis salivarius-bacitracin agar selective for *S. mutans*. The plates were then incubated for 48 h under microaerophilic conditions at 37°C. Following incubation, colonies with morphologic characteristics of SM (0.5 mm raised convex undulated colonies of light blue color with rough margins, granular frosted glass appearance were counted)[24] using a magnifying lens and expressed as number of colony-forming units (CFU) per/ml of saliva. Further identification for SM was confirmed by Gram staining.

Identification of the study sample

The categorization of the children based on the microbiological evaluation was done according to the criteria as follows.[25]

- Class 1: $<10^4$ CFU/ml
- Class 2: 10^4–10^5 CFU/ml
- Class 3: 10^5–10^6 CFU/ml
- Class 4: $>10^6$ CFU/ml.

Finally, 120 children whose CFU counts were within the Class 2 and 3 categories were shortlisted for the study and were randomly assigned to four groups. Each group consisted of 30 children. The children were provided with test materials as follows:

- Group I: TCP (Clinpro™, 3M ESPE, US)
- Group II: CSP (Vantej, Dr. Reddy's Laboratory Ltd, Hyderabad, India)
- Group III: CPP-ACP enhanced with Fluoride (GC Tooth Mousse Plus, Global Care Asia Dental Pte Ltd, Hyderabad, India)
- Group IV: Control (children were asked to follow their routine oral hygiene procedure).

Implementation of the study

Tooth brushing technique (Fone's technique) was demonstrated to the children in the presence of their parents. The children were asked to place an amount of toothpaste (equal to the size of a pea) on a new soft toothbrush which was provided and were asked to brush under parent's supervision twice daily for a month. Salivary samples were collected at intervals of 1 week, 2 weeks, and 4 weeks from the children in all four groups, from the start of the brushing with the respective test materials. The salivary samples were transported, plated, and incubated for 48 h, and the number of CFU/ml of SM in saliva were counted.

Statistical analysis

The result obtained was statistical analyzed using SPSS (version 20; SPSS Inc., Chicago, IL, USA). Chi-square tests were used to assess the differences in SM colony counts at baseline, after 1 week, 2 weeks, and 4 weeks. Kruskal–Wallis test for multiple comparisons was applied. Wilcoxon Signed Ranks test was used to analyze the scores between pre- and post-distribution of dentifrices. $P < 0.05$ was considered statistically significant.

Results

A total of 120 children participated in the study, out of which 65 (54.2%) were males and 55 (45.8%) were females. The mean age of the study population was 72.03 ± 12.52 months. In Group I (TCP), the mean baseline score was 15×10^5 CFU/ml, and it reduced to 6×10^5 CFU/ml and 4.1×10^5 CFU/ml after 1 week and 2 weeks, respectively. After 4 weeks, it increased to 4.5×10^5 CFU/ml. In Group II (CSP) and Group III (CPP-ACP with fluoride), there was a decline in the mean scores from baseline to 4 weeks. In Group IV (Control), the mean baseline score was 13×10^5 CFU/ml which reduced to 12×10^5 CFU/ml, 10×10^5 CFU/ml, and 10×10^5 CFU/ml after 1 week, 2 weeks, and 4 weeks, respectively. At baseline, no significant differences were observed in the mean scores of SM ($P > 0.05$) between the study groups. After 1 week, 2 weeks, and 4 weeks, there was a significant reduction in the mean score of SM ($P < 0.05$) [Table 1 and Figure 1].

Table 2 shows differences in the mean reduction of CFU/ml of SM in saliva. From the start of brushing, there was a statistically significant decrease in SM count (CFU/ml of saliva) in all the test groups as compared to the control group. This reduction in CFU was seen between all the time periods. The maximum reduction

Table 1: Comparison of pre- and post-treatment values of *Streptococcus mutans* of saliva (colony-forming units/ml)

Groups	Mean±SD			
	Baseline ($\times 10^5$ CFU/ml)	1 week ($\times 10^5$ CFU/ml)	2 weeks ($\times 10^5$ CFU/ml)	4 weeks ($\times 10^5$ CFU/ml)
Group I (TCP)	15±11	6±9	4.1±6	4.5±8
Group II (CSP)	17±14	7±8	5±9	4±8
Group III (CPP-ACP with fluoride)	20±12	5±8	1±2	0.5±0.7
Group IV (control)	13±10	12±10	10±9	10±8
*P***	0.200 (NS)	0.001 (significant)	0.001 (significant)	0.001 (significant)

**Chi-square test was used to calculate the *P* value. SD=Standard deviation; CFU=Colony-forming units; TCP=Tricalcium phosphate; CSP=Calcium sodium phosphosilicate; CPP=Casein phosphopeptide; ACP=Amorphous calcium phosphate; NS=Not significant

Table 2: Mean reduction in colony-forming units/ml at various time period

Time period	Group I ($\times 10^5$ CFU/ml)	Group II ($\times 10^5$ CFU/ml)	Group III ($\times 10^5$ CFU/ml)	Group IV ($\times 10^5$ CFU/ml)
Difference between baseline to 1 weeks	9	10	15	1
*P**	0.001 (significant)	0.001 (significant)	0.001 (significant)	0.091 (NS)
Difference between baseline to 2 weeks	10.9	12	19	2
*P**	0.001 (significant)	0.001 (significant)	0.001 (significant)	0.005 (significant)
Difference between baseline to 4 weeks	10.5	13	19.5	3
*P**	0.001 (significant)	0.001 (significant)	0.001 (significant)	0.001 (significant)

*Wilcoxon Signed Ranks test was used to calculate the multiple comparisons, and Kruskal–Wallis test was used to calculate the significance level between the groups. CFU=Colony-forming units; NS=Not significant

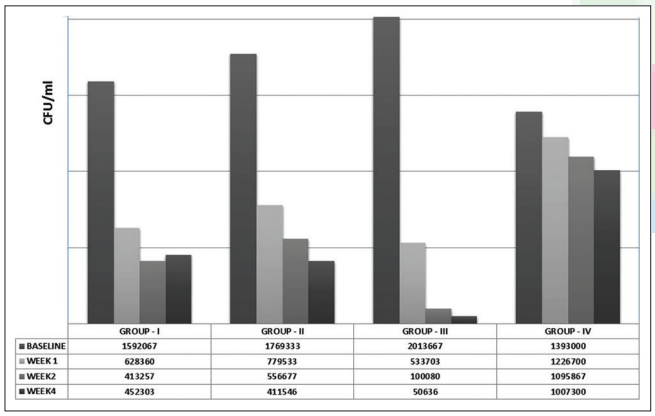

Figure 1: Comparison of pre- and post-treatment values of *Streptococcus mutans* (CFU/ml) of saliva

in the CFU/ml in the saliva of the children was seen in the 1st week after the commencement of the brushing in all the three test groups. Group III children who brushed with CPP-ACP with fluoride demonstrated the maximum reduction of 15 × 10^5 CFU/ml, followed by Group II children who brushed with CSP which showed a reduction of 10 × 10^5 CFU/ml. The children in Group I who brushed with TCP showed a reduction of 9 × 10^5 CFU/ml. The least reduction in the mean difference of the SM counts was (10 × 10^5 CFU/ml) seen in the control group. The reduction in SM counts recorded after 2nd and 4th weeks was lesser compared to the 1st week.

Discussion

In the present study, children who brushed using CPP-ACP with fluoride demonstrated the maximum reduction of SM count over a period of 1 week, 2 weeks, and 4 weeks. Neeser *et al.*[26] demonstrated a restriction of adherence of SM to saliva-coated hydroxyapatite beads by milk casein derivatives. It has been proposed that adsorption of CPP-ACP on enamel causes an increase in the surface net negative charge, thereby influencing the long-range interactions with microbes through the development of repulsive forces.[27] Moreover, CPP-ACP has been considered to modify long-term adhesion by masking the streptococci-related receptors on salivary molecules.[28] A study by Schüpbach *et al.*[29] demonstrated the anticariogenic property of CPP-ACP in the saliva and reported a significant reduction in the total microbial count in saliva. Furthermore, Sun *et al.*[30] reported that a treatment with a greater anticaries potential may be obtained by combining agents that affect the dental hard tissues as well as microbial biofilms. Based on this combination approach, fluoride has been combined with CPP-ACP which not only affects remineralization and demineralization but also controls cariogenic bacteria and disrupts biofilm matrix. Fluoride plays a significant role in dentistry and is influential in the treatment of incipient dental caries as well as prevention for future dental caries. Surface treatment of glass ionomer (GI) and resin-modified GI

with 5% NaF showed drastic reduction in growth of the *S. mutans*.[31]

Reynolds *et al*.[32] investigated the anticariogenicity of CPP-ACP and found that the additive anticariogenic effect obtained with CPP-ACP plus fluoride could relate to fluoride also being incorporated into the CPP-ACP complex. In this way, the CPP could act as an efficient delivery system not only for ACP but also for fluoride. Similarly, in the present research, the highest reduction in the SM level after 1 week may be due to CPP-ACP plus fluoride where CPP could have acted as an efficient delivery system. The reported additive anticariogenic effect of CPP-ACP and fluoride may therefore be attributable to the localization of the novel calcium, fluoride, and phosphate ion nanoclusters at the tooth surface. This localization provides bioavailable fluoride ions to form fluorapatite.[13] Fluoride contributes to caries inhibition in the oral environment by means of both physiochemical and biological mechanisms.[33] A study in preschool children revealed that CPP-ACP reduced *S. mutans* in the plaque after 2 weeks of daily application compared to baseline.[34] Physiochemically, fluoride inhibits demineralization through the formation of fluorapatite enamel. The biologic mechanisms include inhibition of carbohydrate metabolism by SM. As the extracellular pH decreases, protonated fluoride hydrofluoric acid enters SM cell's alkaline cytoplasm and dissociates into H^+ and F^-. The protons will acidify the cytoplasm reducing the pH while the available fluoride interacts with cellular cations and inhibits the cellular process.[35]

CSP (NovaMin) is a bioactive glass in the class of highly biocompatible materials that were originally developed as bone regenerative material.[36] These materials are reactive when exposed to body fluids and deposit hydroxycarbonate apatite, a mineral that is chemically similar to natural tooth mineral[37] and has ionic concentrations close to human blood plasma.[38] When incorporated into a dentifrice, NovaMin particles are deposited onto dentin surfaces and mechanically occlude dentinal tubules.[39] The ability of bioactive glasses to form apatite in body fluids is also used in toothpaste for treating dentine hypersensitivity.[40] However, the present research is focused on the action of CSP on the reduction of SM count in saliva of children. There was a considerable reduction in the SM count after 1, 2, and 4 weeks after the application of the study material in our study.

Allan *et al*.[41] conducted a study on the antibacterial activity of particulate bioglass against supra- and subgingival bacteria. The results from the experiments examining the effect of pH adjustment on the antibacterial activity of bioglass supernates indicated that the antibacterial activity of bioglass supernates was a pH-related phenomenon. High pH solutions have previously been shown to have some antibacterial activities.[42] Dentifrices of high pH have also been shown to be bactericidal.[43] Allan *et al*.[41] concluded that particulate bioglass exerted a considerable antibacterial effect against certain oral bacteria, including those associated with caries and periodontal disease. This antibacterial effect may reduce the potential for bacterial colonization of bioglass. In the current study, the reduction in the SM level may be due to the reduction in the bacterial colonization and high pH.

There is no scientific literature available on the antimicrobial efficacy of TCP. However, TCP has been intensively investigated as a possible bone substitute because of its biodegradable and high osteoconductive properties.[44] Cunha *et al*.[45] conducted a study on the composition of plaque and saliva following a sucrose challenge and use of a TCP-containing chewing gum, where the investigators used a microanalytical technique to examine the anticaries potential of a 2.5% TCP-fortified experimental gum. They suggested that the deposition of a mineral reservoir in plaque and saliva by the experimental gum may help resist the future cariogenic challenge. In the present study, there was a reduction in SM count in the test group which had used TCP, which may be due to the high alkaline pH that may be attributed to the calcium ions in TCP.

There was a certain amount of reduction in the SM count in the control group over the 1-month period. This may be due to the instructions given at the start of the study to all the groups in common, on the brushing technique under parental supervision.[46] However, this reduction was not statistically significant compared with the other three test groups. When intergroup comparison was made in the SM count, the highest reduction was observed in the group which used CPP-ACP with fluoride, followed by CSP, TCP, and the control group. This is in agreement with earlier studies.[18,19,21] The actual mechanism for this reduction is unclear. Further investigations are required to study the mechanism of action of TCP and CSP in reducing the SM counts. A short follow-up period of 4 weeks may be considered as a major limitation of this study.

Conclusions

The following conclusions are drawn from the results of this study.

- The use of CPP-ACP with fluoride, CSP, and TCP twice daily caused a significant reduction in the levels of SM in saliva

- CPP-ACP with fluoride showed the greatest reduction in SM levels in saliva as compared to CSP and TCP.

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Conflicts of interest

There are no conflicts of interest.

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