Original Article

Comparative evaluation of the efficacy of fluoride varnish and casein phosphopeptide – Amorphous calcium phosphate in reducing *Streptococcus mutans* counts in dental plaque of children: An *in vivo* study

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Abstract

Aim: To assess the comparative efficacy of fluoride varnish and casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) complex visa viz. *Streptococcus mutans* in plaque, and thereby the role that these two agents could play in the prevention of dental caries. **Materials and Methods:** A cluster sample of 120 caries inactive individuals belonging to moderate and high caries risk group were selected from 3–5-year-old age group based on the criteria given by Krassee and were randomized to four groups, namely, fluoride varnish – Group I, CPP–ACP complex – Group II, mixture of CPP–ACP complex –Gourp III, and fluoride and routine oral hygiene procedures as control – Group IV. The results thus obtained were analyzed using Statistical Package for the Social Sciences (SPSS) version 16. **Results:** A statistically significant difference in the pre and post-application scores of *S. mutans* ($P < 0.01$) count was observed in all the groups with CPP–ACP plus fluoride group being the most proficient. **Conclusion:** Materials such as fluoride varnish, CPP–ACP, and CPP–ACP plus fluoride protects the tooth structure, preserving the integrity of primary dentition, with the most encouraging results being with CPP–ACP plus fluoride.

Key words: *Casein phosphopeptide–amorphous calcium phosphate (CPP–ACP), fluoride varnish, Streptococcus mutans*

INTRODUCTION

The most important of the dental diseases, which requires prevention as first approach, is dental caries. Although the prevalence has markedly declined, it still poses a major challenge for children and adults. Caries is perceived to be a prolonged imbalance in the oral cavity with alternating episodes of demineralization and remineralization, occurring without any loss of tooth structure.[1,2]

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Streptococcus mutans, one of the most important organism in the plaque biofilm, possesses a unique array of putative cariogenic trait and is the most virulent of the caries producing organisms.[3,4] Longitudinal studies have shown a relative rise of *S. mutans* in plaque samples from tooth surfaces that become carious at a later stage.[4-6] Microbial monitoring has been considered as an alternative method for evaluating current caries activity and future caries risk methods aiming at the estimation of the chief pathogen, i.e., *S. mutans*.

The mainstay of caries prevention revolves around a number of vehicles which have proved themselves to be potent caries inhibiting factors, one of these being fluorides in the form of varnish, whose anticariogenecity has been consistently demonstrated.[7] Fluoride works primarily via topical mechanisms by inhibition of demineralization, enhancement of remineralization at the crystal surfaces, and inhibition of bacterial enzymes.[8] Although it is present in low concentrations, the fluoride accumulated in plaque decreases microbial acid production.

With thorough knowledge on dynamics of caries, demineralization, and remineralization cycles, the quest of mankind for newer materials to overcome the carious challenge has led to the introduction of casein phosphopeptide–amorphous calcium phosphate (CPP–ACP).[2] An amorphous form of calcium phosphate (ACP) stabilized by a phosphopeptide from the milk protein casein (CPP) may be used to localize ACP in dental plaque, maintaining a state of super-saturation with respect to tooth enamel, thus reducing demineralization.[9] Incorporation of CPP into the salivary pellicle substantially reduces the adhesion of *S. mutans*, and this relative selective inhibition would eventually produce a non-cariogenic plaque.[10]

The present study was conducted to compare the efficacy of the fluoride varnish and CPP–ACP complex visa viz. *S. mutans* in plaque, and thereby the role these two agents could play in the prevention of dental caries.

MATERIALS AND METHODS

The present study was conducted in the Department of Pedodontics and Preventive Dentistry between July 2015 and September 2015 in association with the Department of Microbiology, Swargiya Dadasaheb Kalmegh Smruti Dental College and Hospital, Nagpur, with approval from the Ethical Committee, Swargiya Dadasaheb Kalmegh Smruti Dental College and Hospital, Nagpur, and consent from the parents of the children participating in the study.

Sample selection

Of the 568 kindergarten children screened, 280 caries inactive children with complete set of primary dentition, aged 3–5 years, were randomly selected. The children's personal details and details of past medical and dental history including any recent antibiotic and fluoride exposure were obtained.

The children were included in the present study based on the following criteria:

- Caries inactive primary dentition
- No history of antibiotics for the past 3–4 weeks
- No physically and mentally challenged patients or children with any systemic disorders
- No history of fluoride treatment for the past 2 weeks
- Children with any intraoral appliance were not included.

Among the 280 caries inactive children selected, 120 children, who after microbiological evaluation carried out by the first author (95% confidence limit, 7% confidence interval), satisfied the criteria of belonging to the moderate and high caries risk group proposed by Krassee in 1985 were chosen for the study,[11] that is,

Class 0: <10000 CFU/ml (no risk) (CFU: colony forming unit)
Class 1: <100,000 CFU/ml (low risk)
Class 2: 100,000–100,0000 CFU/ml (moderate risk)
Class 3: >100,0000 CFU/ml (high risk)

The nature and objective of the clinical trial as well as the possible side effects were explained and informed consent was obtained from the participants.

Study groups

The children were randomly allocated to four groups, namely, fluoride varnish, CPP–ACP complex, and a mix of CPP–ACP complex and fluoride was painted on the primary teeth of the children belonging to Group I (n = 30), Group II (n = 30), and Group III (n = 30), respectively. A control group (n = 30) was considered to be group IV who were to follow their routine oral hygiene procedures.

Materials

The materials used for this study were Fluor Protector Fluoride varnish (Ivoclar Vivadent, Germany), Tooth Mousse (CPP–ACP) (Global Care Asia Dental Pte Ltd), Tooth Mousse Plus (CPP–ACP + Fluoride) (Global Care Asia Dental Pte Ltd), and Mutans Sanguis Agar (Himedia).

Plaque sample collection

The sample was collected 1–2 hours after brushing to minimize any effect on the growth of the microorganism. The plaque samples were collected from the following four sites using separate toothpicks for the four sites for all the groups:

- Maxillary right molar – buccal surface
- Maxillary incisor – labial surface
- Mandibular incisor – labial surface
- Mandibular left molar – lingual surface.

The four tooth picks were then placed in a single test tube with 1 ml of sterile saline to maintain the accuracy of the number of bacteria present from the time of collection until the processing procedure in the laboratory.

Lottery method of sampling was implemented for allocating the participants into the control group and the study groups. Every participant had an equal chance of being in any of the groups. Groups I, II, III (test groups) and Group IV (control group) consisted of 30 participants each. The participants were blinded to the group to which they belonged.

The plaque samples were obtained from the abovementioned four sites and stored in test tubes containing 1 ml saline. The test tubes were then labeled as per their lot number and placed in an upright position in a cool storage device using dry ice.

Within 30 min of sample collection, the test tubes were transported to the laboratory. The samples were then agitated in a vortex mixture for 45 s in order to disperse bacterial aggregates and to facilitate extraction of bacteria from the tooth pick. The dispersed samples were spread over the culture media plates containing Mutans Sanguis Agar supplemented with Bacitracin (300 units) and 20% sucrose (mitis salivarius-bacitracin Agar), and the plates were incubated under aerobic conditions with a minimum incubation time of 24 h.

On the same day, after obtaining the plaque samples, fluoride varnish, CPP–ACP complex, and a mix of CPP–ACP complex and fluoride was applied to the participants belonging to Groups I, II and III, respectively, by the examiner, and the control group was asked to follow the routine oral hygiene procedures.

Application of fluoride varnish

- Teeth were dried
- With help of a small tufted brush, the varnish was first applied on the lower arch starting with proximal surfaces
- The child was asked to keep the mouth open for 2–3 min before spitting to let the varnish set on the teeth
- The child was requested not to rinse or drink anything at all for 1 h and not to eat anything solid but take liquids and semisolids only till next the morning following application.

Application of CPP–ACP complex

- Excess saliva was removed
- A smear of tooth mousse was applied on the tooth surface using a microbrush or interproximal brush
- It was left undisturbed for 2 min
- The child was then asked to use the tongue to spread the paste throughout the mouth
- The child was encouraged to hold the paste in the mouth for 1–2 minutes, avoiding expectoration
- The child was requested not to eat or drink for 30 min following application.

Application of fluoride and CPP–ACP complex mix

- Excess saliva was removed
- A smear of tooth mousse plus was applied on the tooth surface using a microbrush
- It was left undisturbed for 1–2 min, and the child was asked to distribute the paste throughly with the help of the tongue
- The patient was instructed not to rinse or drink anything at all for 1 h and not to eat anything solid but take liquids and semisolids only till the next morning following application.

The children belonging to group IV (control group) were asked to follow their routine oral hygiene procedures.

Following a period of 24 h, the plaque samples were again obtained from the mentioned sites in the oral cavity of all the participants. These were incubated for the same time and the same methodology was used as before; the same examiner evaluated the results.

After incubation for 24 h, the presence of *S. mutans* was confirmed by the typical colony morphology on the selective culture media and further by gram staining.

The efficacy all the three study groups was assessed by recording *S. mutans* count prior and after the application, and comparing the results with the control group. The *S. mutans* count (CFU/ml) was recorded at 48 h in order to check the efficacy of the action of these

materials over a short period of time. The results were evaluated according to the criteria given by Krassee in 1985.[11]

The results were interpreted by single examiner, who was blinded to the group division. The *S. mutans* growth was identified as raised colonies sideways against a light or with a magnifying glass. To differentiate *S. mutans* colonies and epithelial cells on the culture media, a gloved finger was passed along it to identify the smooth and rough colonies. Only the rough colonies were allotted as the growth of *S. mutans*.

RESULTS

A considerable difference was documented in all the groups in the pre application and post-application scores of *S. mutans* ($P < 0.01$) [Table 1] using Pearson's Chi-square test. The intergroup comparison using Wilcoxon-matched pairs signed ranks test proved CPP–ACP plus fluoride group to be the most proficient [Table 2].

Table 1: Cross tabulation of pre and post-application scores for each study group

Pre-treatment scores	Post-treatment scores				*P* value**
	Class 0	Class 1	Class 2	Class 3	
Group I					
2	6	9	1	1	0.01 (sig)
3	1	3	6	3	
Group II					
2	8	2	4	0	0.01 (sig)
3	3	10	1	2	
Group III					
2	11	1	0	0	0.0009 (sig)
3	4	11	3	0	
Group IV					
2	0	1	14	1	0.0007 (sig)
3	0	1	3	10	

***Pearson's Chi-square test was used to calculate the P value*

Table 2: Comparison of mean scores between pre-application and post-application for each study group

Study groups	Mean±SD			*P* value*
	Pre-application	Post-application	Change	
Group I	2.4±0.5	1.3±1.0	1.1±0.8	<0.0001 (sig)
Group II	2.5±0.5	0.9±0.9	1.6±0.9	<0.0001 (sig)
Group III	2.6±0.5	0.6±0.7	2.0±0.5	<0.0001 (sig)
Group IV	2.5±0.5	2.3±0.6	0.2±0.5	0.12 (ns)

**Wilcoxon-matched pairs signed ranks test was used to calculate the P value*

DISCUSSION

The structure of enamel is unique, in that it has no residual cellular components that effect repair when enamel is damaged by a cariogenic episode.[12] Demineralization and remineralization (repair or healing) of enamel are continual and constant processes occurring on the availability of cariogenic flora and refined carbohydrates.[1,12,13] The plaque biofilm encloses numerous microenvironments that can be disrupted through chemomechanical systems[13] such as applications of topical fluoride, CCP–ACP, and tooth brushing.

The plaque biofilm facilitates the attachment and spread of *S. mutans*, the bacteria most implicated in caries. Plaque fluid, which forms the plaque system, can harbor more concentrations of fluoride, calcium, and phosphate complexes than any other fluid. Hence, in this study, the effect of fluoride varnish and CPP–ACP on *S. mutans* count in plaque of caries inactive individuals was analyzed.

Vogel and Ekstrand[14] found that there exist a marked variation between the plaque fluid fluoride concentrations at various sites of the oral cavity. Considering this fact, plaque samples were simultaneously obtained from different sites of oral cavity.

In the present study, Krasse criteria was used as a tool for evaluating the pre and post-application microbial count because it is a simple, non-invasive and patient friendly technique.[3,4,11]

In the present study, brushing was advised with toothpaste containing fluoride prior to sample collection because it enhances the uptake of CPP–ACP.[15] Topical fluorides significantly contributes in reducing the prevalence of caries, and it has been established that its anticaries efficacy against plaque micro-organisms and remineralization potential substantially increases when available in high concentrations in oral cavity locally.[16,17]

A significant difference observed in the pre and post-application scores [Table 1] ($P < 0.01$) of Group I, where the mean score before treatment being 2.4 ± 0.5 decreased to a mean score of 1.3 ± 1.0 after the application of fluoride varnish, emphasizing the efficacy of fluoride against Streptococcus.

Hydroxyapatite crystals, when pre-treated with fluor protector, produced significantly reduced lactic acid formation by *S. mutans*.[18] Hence, the reduction of bacterial count in this study could be due to high concentration of fluoride from fluor protector, which might have entered the bacterial cell and resulted in the inhibition of various cellular processes such as:[19,20]

- Inhibition of enolase that indirectly affects the formation of ATP, which is central to cell maintenance and growth
- Inhibition of H+/ATPase in the bacteria which compromises the maintenance of cellular pH and makes the internal environment more acidic and unsuitable for other enzymes to act
- Inhibition of exogenous glycerol uptake into lipoteichoic acid which is believed to play a crucial role in membrane stability as well as in colonization of *S. mutans* on hydroxyapatite
- Reduction of the peptidoglycan macromolecule in the cell membrane and thereby causing partial lysis of cell membrane.

However consumption of fluoride above the therapeutic dose over an extended period of time particularly in younger age group may lead to dental fluorosis.[17]

To overcome the drawbacks of fluorides, one of the newer materials that have shown effective protection against caries by promoting lesion remineralization and inhibiting enamel demineralization is CPP–ACP.

Caseinophosphopeptides inhibit dental caries lesions by influencing the demineralization/remineralization process of dental enamel. The active CPP is a naturally occurring molecule that can bind to calcium and phosphates and at the same time stabilize ACP. In the oral cavity, calcium and phosphate ions are released from CPP as the pH in the plaque decreases resulting in supersaturation which reduces demineralization and promotes remineralization.[7,9]

A mean decrease of 1.6 ± 0.9 for group II which was statistically significant ($P < 0.0001$) was observed. The mean change in Group II was significantly higher than the mean change in Group I ($P < 0.05$) showing that CPP–ACP had a better anticariogenic potential compared to fluoride varnish.

Caseinophosphopeptides stabilizes ACP, which in turn localizes ACP in dental plaque, acting as large calcium reservoir within plaque that slows the diffusion of free calcium ions providing a source of calcium for remineralization, and thus restricting mineral loss during a cariogenic episode.[21] Therefore, the mechanism of anticariogenicity for CPP–ACP is that this bioactive peptide substantially increases the level of ACP in plaque, thus depressing enamel demineralization and enhancing remineralization. Because the concentration of CPP–ACP in contact with tooth enamel increases so does remineralization. Reynolds[9] showed an increased concentration of free and bound calcium in 0.5 and 1.0% CPP–ACP preparations, which was most effective at remineralization of the dental enamel.

There is reduced enamel subsurface demineralization when enamel plaque is exposed to solutions of tryptic peptides of casein. Thus, incorporating casein peptides into enamel plaque increases the plaque's content of calcium and phosphate.[2] The tryptic peptides responsible for caseinate's anticariogenic activity are the calcium phosphate stabilizing CPPs. These CPPs contain a specific sequence, which markedly increases the apparent solubility of calcium phosphate by stabilizing ACP, forming solutions that are supersaturated with respect to calcium phosphates.

In a caries study, using 0.5% weights per volume (w/v) solution of CPP–ACP nanocomplexes with a 500 ppm fluoride solution, the anticariogenecity of CPP–ACP and fluoride together was additive, when compared to either CPP–ACP or fluoride alone.[22]

The advantage of using CPP–ACP complexed with fluoride is the simultaneous availability of calcium, phosphate, and fluoride. The calcium phosphate in these complexes is biologically available for remineralization of subsurface lesions in tooth enamel. In addition, in an *in vitro* study, Reynolds *et al*. found that the enamel remineralization potential of mouthwash containing CPP–ACP and 220 ppm fluoride was superior than CPP–ACP and fluoride mouth rinse.[23]

The distribution of participants according to the *S. mutans* score pre-application as 12 in class 2 and 18 in class 3 in group III for application of CPP–ACP plus fluoride ($P = 0.60$) changed to 15, 12, 3, and 0 in class 0, 1, 2, and 3, respectively ($P < 0.0001$). The mean score before treatment was 2.6 ± 0.5 which decreased to a mean score of 0.6 ± 0.7, drawing attention to the fact that CPP–ACP and fluoride together is more proficient than either of the materials used independently, which was also observed by Sudjalim *et al*.[24] and Erdam *et al*.[25]

Further, in the present study, no significant differences were reported among experimental and control groups in relation to incidence of adverse reactions. This is in accordance with the work of Rao *et al*.[26] and Sitthisettapong *et al*.[27]

Well-documented evidence of fluorides reducing caries by various mechanisms has been clearly established, however, its hazardous effects cannot be overlooked.[16,17] To overcome these limitations, researchers have developed various nonfluoride caries preventive and remineralizing agents including CPP–ACP and CPP–ACP plus fluorides. Literature regarding the caries preventive and remineralization action of CPP–ACP and CPP–ACP plus fluorides is scarce, and the current review papers and meta-analysis focus on the requirement of further well-designed clinical trials and also advocate that the use of these materials must be encouraged by practicing dentists in day-to-day oral hygiene practices and dental materials.[2,7,8,21,28-30]

The results of the present study indicate that the use of the materials fluoride varnish, CPP–ACP, and CPP–ACP plus fluoride prevents the loss of tooth structure, preserving the integrity of primary dentition with the most encouraging results with CPP–ACP plus fluoride.

CONCLUSION

With the ever changing scenario of preventive dentistry, the efficacy of newly introduced materials such as CPP–ACP in preventing enamel demineralization against the established material fluoride warrants further clinical trials. Therefore, the present study using fluoride varnish, CPP–ACP, and CPP–ACP plus fluoride was conducted, and the results revealed that there was a statistically significant reduction in the counts of *S. mutans* in the plaque biofilm after a period of 24 hours.

Although the clinical trials on CPP–ACP and CPP–ACP plus fluoride are still in the infancy stage, the results of the present study stress the continued need for the long-term efficacy of these materials in changing the dynamics of dental caries towards remineralization, consequent to significant reduction in the counts of cariogenic bacteria both in children and adults.

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Conflicts of interest

There are no conflicts of interest.

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