The Effects of Three Different Pastes on Enamel Caries Formation and Lesion Depth Progression- An In Vitro Study

Ravi V Shirahatti, Anil V Ankola, Nagesh L, Seema Hallikerimath

ABSTRACT

Prevention of initiation and interruption in progression of early lesions are the desirable modes of caries management. Fluoride dentifrices and casein phosphopeptides are known to inhibit demineralization and enhance remineralization. The present study was conducted with the objective to investigate the effects of fluoridated dentifrice, non-fluoridated dentifrice and casein phosphopeptide amorphous calcium phosphate paste on enamel caries formation and lesion depth progression. 228 tooth sections derived from extracted human permanent molars were used. The study was carried out in 3 phases where in each phase the enamel sections were treated for 14 days twice a day for 3 minutes with respective agents, followed by acid demineralization using 0.05 M acetic acid resulting in lesions. The depths of the lesions were measured using stereomicroscope. The mean lesion depths of three test groups were compared with each other as well as with control group at the end of each phase. Comparison of mean lesion depths of different groups was carried out using analysis of variance (ANOVA) and student’s unpaired-t-test. The fluoridated dentifrice group had significantly lesser mean lesion depth than control group in all the three phases (P<0.05). In second phase, non-fluoridated dentifrice and CPP-ACP paste groups had significantly lesser mean lesion depths than control groups (P<0.05). The study demonstrated that twice a day application of fluoridated dentifrice provides substantial protective effect against lesion formation and lesion depth progression. The study could not demonstrate any additional ability of CPP-ACP paste in reducing lesions depth progression and effect was similar to non-fluoridated dentifrice group.

Key words: Phosphopeptides; Toothpaste; Dentifrices; Fluorides; Casein; CPP-ACP; In Vitro; Dental Caries; Tooth Demineralization; Dental Enamel

Fluoride is a preventive agent that has almost mesmerized the dental research. It is one of the elements categorized by Navia(1) as “strongly cariostatic”. Although fluoride has had a profound effect on level of caries prevalence, it is far from complete cure. It is unlikely that there is any concentration of fluoride, which will eliminate caries totally.(2) High fluoride strategy cannot be followed in most instances to avoid potential for adverse effects due to over exposure to fluoride. Thus, there is scope for agents, which may be used with fluoride to enhance anticaries activity.(2) This need has redirected dental research to develop novel preventive agents that can act as an adjunct to fluoride or independent of it. Casein phosphopeptide is one such agent that has been proposed to have anti-cariogenic properties. Phosphopeptides of casein are produced from a tryptic digest of milk protein ‘casein’ by aggregation with calcium and phosphate. Casein is said to have an ability to localize amorphous calcium and phosphate with respect to tooth enamel, depressing demineralization and enhancing remineralization.(3) Pastes incorporating casein phosphopeptide are currently available. Previous studies have
focused on casein phosphopeptide incorporated in sugar free gums, lozenges and mouthwashes.(4,5,6) But there is lack of information on effect of casein phosphopeptide incorporation to regularly used dentifrices on enamel caries. Hence an in vitro study was conducted with a purpose to compare the effects of this newer material with the more commonly used fluoridated and non-fluoridated dentifrices. The study was started with a null hypothesis that 'there is no difference in the depths of the artificial enamel lesions, when the mean lesion depths of the three test groups are compared between one another as well as when they are compared with the non-application control.' The study was conducted with the objectives to investigate the effects of fluoridated dentifrice, non-fluoridated dentifrice and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste on enamel caries initiation and progression.

**Materials and methods**

An in vitro study was designed and conducted in the Department of Preventive and Community Dentistry with the assistance from Department of Oral Pathology, KLES’s Institute of Dental Sciences, Belgaum. Ethical clearance for the study was sought and obtained from ethical committee for the use of human extracted teeth. The sample size consisted of 228 tooth quarters derived from 57 teeth. This sample size was adequate for the study as determined previously from the pilot study. Extracted teeth were obtained from Department of Oral Surgery, KLES’s Institute of Dental Sciences, Belgaum. The following inclusion and exclusion criteria were followed to select the teeth for this study. Inclusion criteria included extracted, macroscopically caries free, human permanent molars were included in the study. Exclusion criteria followed was that the teeth having intrinsic stains, dental caries, gross surface defects like pits and cracks (on the Buccal, Lingual and Proximal surfaces) and gross hypoplasia were excluded. Those teeth, which were having stains even after a thorough prophylaxis, were excluded from the study. Rest of the teeth fulfilling all the selection criteria were stored in antifungal solution containing 0.1% Thymol until the experimental procedure was initiated. This procedure also helped to prevent dehydration.

Each tooth was sectioned longitudinally along the marked plane of sectioning yielding the following four tooth quarters:

- Mesio-buccal (MB)
- Disto-buccal (DB)
- Mesio-lingual or Mesio-palatal (ML)
- Disto-lingual or Disto-palatal (DL)

Crown preparation burs and air turbine hand piece were used for tooth sectioning. Each tooth quarter was reduced to a thickness of three millimeters using grinding procedure. Each tooth quarter was tagged indicating its tooth and section code. A total of 228 tooth quarters obtained from 57 teeth were coded and segregated according to quadrants.

One tooth quarter from each tooth was assigned to one of the following study groups:

- **Group 1 - Control Group**
- **Group 2 - Non-fluoridated dentifrice treatment group**
- **Group 3 - Fluoridated dentifrice treatment group**
- **Group 4 - “CPP-ACP” paste treatment group**

The coding and systematic allocation was done to serve following purposes:

- During the measurement of lesion depth the coding system helped to achieve blinding of the investigator.
- One section from each tooth was allotted to each study group
- All the study groups received equal number of Mesio-buccal, Disto-buccal Mesio-lingual and Disto-lingual tooth quarters.
- Each of the study group received sections of teeth from all the four quadrants.

All the coded tooth sections were subjected to enamel window formation before the first phase of study was initiated. Each enamel surface on the tooth quarter was marked with an area of 3 x 3mm. Rest of the area of the tooth quarter was covered with acid resistant nail varnish. This was done to limit the area of paste application and to produce lesions only in window area. This would help in lesion depth measurement compared to unaffected area covered by nail varnish. After application of nail varnish each section was placed in a bottle containing 30 ml artificial saliva. These tooth quarters, three phases of study.

**First phase**

Each of the test groups [Group 2, 3 and 4] was treated with the respective agents. The exposed enamel windows received the paste application twice a day. Each time, the corresponding pastes were applied for a period of three minutes. Control group [Group 1] was not treated with any agent, and the sections were allowed to remain in artificial saliva only. This first phase of application was done for a period of 14 days. Fresh artificial saliva was prepared and was changed in every 72 hours. At the end of fourteen days all the tooth quarters were ready for acid demineralization. Immediately after acid dissolution, all the tooth quarters were washed in distilled water and placed in a plastic cover. The tags with identification codes served as identification for each tooth quarter. The acid resistant nail varnish was removed using acetone. The lesion depths at initiation were measured under stereomicroscope. The depth of the lesion was observed in relation to the sound intact tooth surface. The depth was measured at three designated locations one in the centre of lesion, second at the incisal or occlusal end of the lesion and third at the cervical end of lesion. The depths of lesions were measured using a micrometer eyepiece. The eyepiece had calibrations at regular intervals giving readings in units. All the three readings were converted to measurements in microns. An average of the three readings was taken to determine the lesion depth of each lesion.
Second phase
After the lesion depths were measured at end of first phase, the tooth sections were reapplied with nail varnish leaving the 3X3 mm window open and the caries like lesions in each test group was treated again for 14 days with respective tests agents twice a day for 3 minutes. Following the second phase of paste application the lesions were dipped in acidic medium for six days as described in first phase for lesion progression. This was followed by removal of nail varnish and lesion depth measurement for a second time using stereomicroscope.

Third phase
The whole procedure as described in first and second phases was repeated again by a third phase of 14 days of paste application and third phase of acid demineralization (second lesion progression). The final lesion depth measurement was done at the end of third phase.

Statistical tests used included Weighted Kappa statistics for repeatability of readings where we observed a Kappa value of 0.80, which depicts the good repeatability of the readings taken during the study period. Analysis of variance (ANOVA) test was used in order to know the statistical significance of the variability within and in between the four groups. A p-value of less than 0.05 was taken as statistically significant. Students unpaired ‘t’ test was applied when a significant “in between group” variation was observed on use of analysis of variance (ANOVA). Student’s unpaired t-test was applied to know whether the differences in the average or mean lesion depths of any two groups being compared were statistically significant or not. A p-value of less than 0.05 was taken as statistically significant.

### Results

#### Table 1: Mean lesion depths of the four groups (ANOVA)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean lesion depths in microns ( \pm ) standard deviation</th>
<th>First phase</th>
<th>Second phase</th>
<th>Third phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>149.590 ( \pm ) 46.435</td>
<td>331.111</td>
<td>426.666</td>
<td></td>
</tr>
<tr>
<td>Non-fluoridated dentifrice</td>
<td>135.243 ( \pm ) 50.492</td>
<td>286.90</td>
<td>414.152</td>
<td></td>
</tr>
<tr>
<td>Fluoridated dentifrice</td>
<td>113.060 ( \pm ) 50.705</td>
<td>237.309</td>
<td>339.883</td>
<td></td>
</tr>
<tr>
<td>Casein phosphopeptide-amorphous calcium phosphate paste</td>
<td>140.662 ( \pm ) 52.560</td>
<td>285.61</td>
<td>387.368</td>
<td></td>
</tr>
</tbody>
</table>

Single factor analysis of variance for in between group comparison

- F-value-5.494
- F-value-9.0363
- F-value-5.615

- P-value-0.001166
- P-value-0.00011
- P-value-0.000948

Very Significant

Highly significant

Highly Significant

#### Table 2: Comparison of control group with the other three test groups (students ‘t’ test)

<table>
<thead>
<tr>
<th>Test groups compared with the control group</th>
<th>Percentage difference in mean lesion depths when compared with control</th>
<th>First phase</th>
<th>Second phase</th>
<th>Third phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fluoridated dentifrice</td>
<td>9.59%</td>
<td>13.35%</td>
<td>2.93%</td>
<td></td>
</tr>
<tr>
<td>P-value 0.1171</td>
<td>p value -0.0117</td>
<td>Significant</td>
<td>Not Significant</td>
<td></td>
</tr>
<tr>
<td>Not Significant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoridated dentifrice</td>
<td>24.42%</td>
<td>28.33%</td>
<td>20.33%</td>
<td></td>
</tr>
<tr>
<td>P-value - 0.000109</td>
<td>p value -0.000006</td>
<td>Highly Significant</td>
<td>P-value 0.000258</td>
<td>Highly Significant</td>
</tr>
<tr>
<td>Highly Significant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein phosphopeptide-amorphous calcium phosphate paste</td>
<td>5.968%</td>
<td>13.74%</td>
<td>9.21%</td>
<td></td>
</tr>
<tr>
<td>P-value 0.3385</td>
<td>p value -0.0120</td>
<td>Significant</td>
<td>P-value 0.0791</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Not Significant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Table 3: Intra-comparison of lesion depths in between the three test groups (student’s ‘t’ test)

<table>
<thead>
<tr>
<th>Group compared</th>
<th>Percentage difference in mean lesion depths in first phase</th>
<th>Percentage difference in mean lesion depths in second phase</th>
<th>Percentage difference in mean lesion depths in third phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoridated dentifrice group with non fluoridated dentifrice group</td>
<td>16.40%</td>
<td>17.28%</td>
<td>17.93%</td>
</tr>
<tr>
<td>P-value 0.021029</td>
<td>P-value 0.007546</td>
<td>P-value 0.002043</td>
<td>Very Significant</td>
</tr>
<tr>
<td>Significant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoridated dentifrice group with CPP-ACP paste group</td>
<td>19.62%</td>
<td>16.91%</td>
<td>12.25%</td>
</tr>
<tr>
<td>P-value 0.005153</td>
<td>P-value 0.011369</td>
<td>P-value 0.043906</td>
<td>Significant</td>
</tr>
<tr>
<td>Very Significant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non fluoridated dentifrice group with CPP-ACP paste group</td>
<td>3.85%</td>
<td>0.45%</td>
<td>6.46%</td>
</tr>
<tr>
<td>P-value 0.5756</td>
<td>P-value 0.94416</td>
<td>P-value 0.24113</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Not Significant</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The present study utilized an in vitro model to test the effects of periodic application of three commercially available agents namely; non-fluoridated dentifrice, fluoridated dentifrice and CPP-ACP paste on enamel sections followed by in vitro acid challenge. The first phase of the present study represented lesion initiation and the next two phases represented lesion progression. The mean lesion depths of artificial lesions in each of the test and control groups were compared for all the three phases. Artificial saliva composition used in the present study was similar to that used by John M. Hicks and Catherine M. Flaitz(7) and similar compositions were used by Joyston-Bechal S, Kidd EA also.(8) The acidic medium used in this study was acetic acid at pH 4.5 similar to that used by ten Cate JM, Duijsters PP.(9) Acid resistant varnish was used in the present study to produce enamel windows similar to that used by Thaveesangpanich and coworkers.(10) The present study used single section technique as used by Donly KJ and colleagues(11) that allows measurements of exactly the same tissue before and after treatment, which helps investigators to observe and quantitatively measure changes in lesion characteristics.

In the first phase of the present study sound enamel windows were treated with respective test agents. Result showed that fluoridated dentifrice treated group had the least mean lesion depth. Fluoridated dentifrice group had 24.42% lesser mean lesion depth compared to control group John M. Hicks and Catherine M. Flaitz(7) conducted a similar study designed with one initiation and two progression stages. Similar to the present study, a 30% reduction in mean lesion depth in fluoridated dentifrice group compared to control group was observed by John M. Hicks and Catherine M. Flaitz(7) who used thrice a day application regimen. In contrast, in the present study, we followed twice a day application regimen. Rolla G. Ogaard B and Cruz RA(12) also stated that daily application of fluoridated toothpaste results in formation of a calcium fluoride “reservoir” on tooth surface, which inhibits demineralization by release of fluoride on subsequent acid challenge. This inhibition to demineralization can be brought about by fluoride levels as low as 0.014-0.024 ppm as reported by Page DJ(13), and several other investigators (Margolis and coworkers ; Lynch RJ et al).(14,15)

Fluoride dentifrice groups in the present study also showed lesser mean lesion depths at first progression similar to our study, John M. Hicks and Catherine M. Flaitz(7) also noted a 27% reduction in fluoride group compared to control group at first lesion progression stage. Ulrich Klein and coworkers(16) reported a 29% reduction in lesion depth progression on one minute pretreatment with 10% stannous fluoride paste. Even higher reductions in lesion depth progression than that observed in the present study have been reported. Featherstone JDB and co-workers(17) observed that 5 minutes daily application of test solution of 62 mg/L and 178 mg/L of fluoride reduced demineralization up to 80% and 90% respectively. Argenta RM(18) and colleagues also observed that fluoride in solution showed a dose response effect in reducing enamel demineralization. The reason for this phenomenon could be due to the nature of artificial carious lesions to readily acquire fluoride as observed by Mellberg JR and Chomichi(19) in an in vivo study. Such incorporated fluoride in lesion could be released during subsequent acid challenge that inhibits further demineralization as observed by Lynch RJM, Navada R and Walia R.(15)

In the third phase of the present study also, the fluoridated dentifrice group showed lesser mean lesion depth compared to control group (Table 8) and this difference was statistically significant (Table 9). A 20.33% lesser lesion depth was observed in the third phase of the study. Similarly a 27% lesser lesion depth in fluoridated paste group compared to control group was observed in second lesion progression stage by John M. Hicks and Catherine M. Flaitz.(7) In all the three phases of the present study fluoridated dentifrice group had lesser lesion depth than non-fluoridated dentifrice group (Table 2, 5 and 8), which was statistically significant. A higher reduction than that observed in the present study was observed by Page and co-workers(13) who reported in a study that use of 1000 ppm and 1500 ppm of fluoridated dentifrice reduced enamel demineralization compared to non-fluoridated group. In another study conducted by Page DJ(13), five minutes exposure to 1000 ppm fluoridated dentifrice exhibited resistance to demineralization by 60.7% and 100 ppm fluoride dentifrice could resist demineralization for up to 50.5%. This higher reduction observed compared to our study could be due to the longer duration of paste application and shorter duration of acid dissolution.

Reduction in demineralization in fluoride dentifrice group compared with non-fluoridated dentifrice has also been observed in in situ studies utilizing intra-oral models. Duggal MS(20) in an in situ study with cross over design observed that fluoride-free pastes lost significantly higher mineral compared to fluoridated pastes. Paes Leme et al (21) observed similar results. Similarly Donly KJ et al(11) also observed that subjects who brushed with fluoride dentifrice lost lesser minerals in lesions adjacent to restoration.

The non-fluoridated dentifrice group in the present study showed lesser lesion depths compared to control group in all the three phases. But the difference was not significant for first and third phases .Brudevold F and co-workers(22) observed that several salts of calcium, sodium, potassium and strontium used as mouth rinse could reduce enamel demineralization in an intra oral model. Different salts reduced the demineralization to different degrees and this was termed as “common ion effect”. An effect similar to the aforementioned
“common ion effect” could have been one of the possible reason for the reduced lesion depth observed in non-fluoridated dentifrice group compared to control group in second phase of our study.

The third agent tested in the present study was the periodic application of paste containing casein phosphopeptide amorphous calcium phosphate (CPP-ACP). CPP-ACP is proposed to have anti-cariogenic potential by depressing demineralization and enhancing remineralization according to a review by EC Reynolds.(3) In the first phase of the present study CPP-ACP group had lesser lesion mean depth compared to control group which was not statistically significant (Table 3). In contrast to the present in vitro study, Roberts AJ(2) reported a significant reduction in enamel demineralization when volunteers used a dentifrice containing 5% CPP and wore an in situ appliance with sound enamel. This could be related to the in vitro presentation of the study, which is in contrast to the aforementioned in situ study that showed a reduction. The presence of plaque, bacteria and saliva, observed in the in situ condition could have significantly influenced the process. The literature points out that CPP can bind to oral bacteria like streptococcus mutans as reported by Rose RK.(23) In an in situ or in vivo model CPP can thus act by these aforementioned mechanisms, which were not possible in an in vitro condition. This could be the possible reason why the CPP-ACP group did not show significant resistance to lesion depth formation, in first phase.

But during the second phase CPP-ACP groups did show 13.74% lesser mean lesion depth than control group which was similar to 13.35% lesser lesion depth observed in non-fluoridated dentifrice group. Roberts AJ(2) reported that enamel pretreated with CPP showed greater retention of calcium than control group to subsequent demineralization. Kanako Yamaguchi and coworkers(24) observed significantly less demineralization of bovine enamel treated with casein phosphopeptide-amorphous calcium phosphate paste. This study used twice a day 10 minutes application of the paste on bovine enamel and an ultrasonic device was used to measure demineralization. In contrast to this we applied the paste twice a day for 3 minutes and demineralization was determined using lesion depth, in our study. Lesser lesion depth in CPP-ACP group in second phase of the present study can also be attributed to the remineralizing ability of casein phosphopeptide paste when applied to the lesions. In the third phase of this study, CPP-ACP group showed a reduction of 9.21% compared to control group but this was not statistically significant. Similar results were obtained by an in vitro study by Lennon AM and coworkers(25) who noted that twice a day application of CPP paste or use of 250 ppm fluoridated dentifrice group did not show any significant reductions in mean lesion depth. Though the acidic condition used by Lennon AM and coworkers (25) was more severe (1% citric acid at pH 2.3) than the present study (acetic acid at pH 4.5) similar results were observed.

The present in vitro study demonstrated that fluoride dentifrices provide a substantial protective effect against lesion formation and lesion progression. Twice a day fluoride dentifrice application substantially influenced the dissolution of sound enamel, as well as enamel with lesions. Value of fluoridated dentifrices in prevention of dental caries has also been confirmed by epidemiological data of the reports of decline in dental caries prevalence, concurrent with rise of fluoridated toothpaste.(26) These findings also have dental health education implications. Dental health education should be directed more towards ensuring tooth brushing with a fluoride containing tooth paste twice a day rather than focusing only on dietary restrictions which is more difficult to achieve.(20) The present study showed some effect of CPP-ACP in reducing lesion depth. But the in vitro model used here was not able to demonstrate emphatically the role of CPP-ACP twice a day application in resisting lesion depth initiation and progression. Different results may be expected in an in situ or in vivo situation where CPP-ACP can bind to oral bacteria (23) and be released from oral reservoir.(27) Longitudinal caries incidence studies can be an alternative.

Conclusions
It can be concluded from the present study that twice a day periodic application of 1000 ppm fluoridated dentifrice could significantly reduce the depth of an enamel lesion produced subsequently by acid challenge. Use of non-fluoridated dentifrice and also the use of paste incorporated with casein phosphopeptide amorphous calcium phosphate can reduce the progression in depths of enamel lesions when applied to early lesions. The resistance to progression of lesion depth diminishes when the agents are applied to more progressed enamel lesions. The conclusions drawn in the present in vitro study need to be substantiated by longitudinal caries incidence studies and in situ studies especially with respect to CPP-ACP incorporated paste compared with the other commonly used fluoridated and non-fluoridated dentifrices.

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Disclaimer
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