Enhanced enamel benefits from a novel fluoride toothpaste

Andrew Joiner, Fred Schäfer, Kate Hornby, Mark Long, Margaret Evans, Tim Beasley and Pam Abraham
Bebington, UK

**Objectives:** Through the understanding of tooth enamel science and insights into the beneficial role calcium can play in the caries process, a novel fluoride toothpaste has been developed containing micro-calcium. This paper describes a series of *in vitro* studies to assess delivery of the micro-calcium to a plaque biofilm, delivery of radiolabelled micro-calcium to subsurface enamel lesions and the rehardening of acid softened enamel by this novel toothpaste. Two clinical studies evaluated the delivery of calcium to the mouth.

**Methods:** Uptake of micro-calcium to a plaque biofilm was assessed using a Calgary Biofilm Device and measuring the calcium levels delivered to the biofilm from the micro-calcium containing toothpaste, a calcium carbonate toothpaste, a silica toothpaste or water controls. Sound and subsurface enamel lesions were treated with $^{45}$Ca labelled micro-calcium toothpaste in an *in vitro* pH cycling study and the uptake of labelled calcium determined. Acid softened enamel specimens were treated with either the micro-calcium containing toothpaste, a calcium carbonate toothpaste or a non-fluoride silica toothpaste in an *in vitro* remineralisation protocol and the changes in surface microhardness measured. Calcium delivery *in vivo* was determined in two double-blind, randomised cross-over studies. Subjects brushed their teeth for one minute with either the micro-calcium containing toothpaste or a silica toothpaste. Immediately after brushing and at fixed time intervals up to one hour, unstimulated saliva samples were taken and the total calcium concentration determined.

**Results:** Significantly (p<0.05) more calcium was delivered to a plaque biofilm from the micro-calcium containing toothpaste than the controls. The radiolabelled micro-calcium study demonstrated the uptake of calcium to subsurface enamel lesions. In the remineralisation study, acid softened enamel became significantly harder (p<0.05) following treatment with the micro-calcium containing toothpaste compared to the control toothpastes. It was shown in the two clinical studies that more calcium was delivered to the mouth following the use of the micro-calcium containing toothpaste than compared to the silica toothpaste. The product differences were in excess of 50% and were of statistical significance (p<0.001).

**Conclusions:** The studies show that the new toothpaste containing micro-calcium delivered elevated levels of calcium to the mouth, promoted enhanced remineralisation of demineralised enamel lesions and thus can help repair early signs of tooth decay.

*Key words: Calcium, remineralisation, caries, enamel, fluoride.*
reduce the dissolution of sound enamel and significantly increase the uptake of calcium to demineralised enamel\(^7,8\). In addition, clinical studies have demonstrated an inverse relationship between fluoride concentration in saliva and/or dental plaque and the prevalence of caries levels\(^9,10\).

Calcium and phosphate are necessary components for the maintenance of strong and healthy teeth. These are found in saliva and form part of the natural defence against caries by influencing the de- and re-mineralisation processes that can occur in teeth. The ionic activities of calcium and phosphate in plaque fluid and its pH are also important in determining the stability of the tooth surface under a cariogenic challenge\(^11\). Under normal circumstances, plaque fluid is supersaturated with respect to enamel minerals and its pH is close to neutral\(^12\). However, periods of undersaturation can occur, usually following meals, where the plaque bacteria metabolise sugars and carbohydrates to produce organic acids which in turn lower the pH at or near the tooth surface\(^13\). Under these conditions an instability in the enamel mineral phases can occur and this results in a net flow of calcium and phosphate ions from the tooth to the surrounding fluid. The dissolution will continue until the pH and calcium and phosphate activities reach levels where the enamel minerals are stable again. The extent of dissolution depends on a number of factors including pH, the types and amounts of acids produced, and the ionic activities of calcium and phosphate\(^14-16\). When conditions return to supersaturation, it is possible for new minerals to be precipitated, replenishing the minerals that were lost during periods of undersaturation\(^17\).

The levels of calcium and phosphate in saliva and plaque have been shown to be inversely associated with caries incidence and low levels of plaque calcium as a predictor of future caries\(^18-20\). Similar findings have been reported from epidemiological studies which provide strong evidence that caries incidence is inversely related to calcium concentration in saliva\(^21\). One reason put forward for this correlation is that increased calcium concentration in saliva will reduce solubility of enamel on the one hand and also increase the driving force for remineralisation of the early stages of enamel demineralisation. Margolis and Moreno\(^22\) found that the degree of saturation with respect to enamel (DS\(_{en}\)) was lower in the plaque samples of caries-positive subjects as compared to plaque samples from caries-free subjects following a cariogenic challenge. Similar observations were made in plaque fluid samples of caries prone subjects\(^23,24\). The magnitude of DS\(_{en}\) is dependent on the available calcium and phosphate ions and theoretically small changes in calcium levels will have a greater effect than similar changes in phosphate levels\(^25\). This has been confirmed via in vitro mechanistic studies with calcium being approximately twenty times as potent as phosphate in inhibiting enamel dissolution through increased DS\(_{en}\)\(^26\). Thus extended elevation of plaque levels would be an effective means of reducing undersaturation during a cariogenic challenge\(^25\).

The relative importance of calcium and phosphate and the rate at which these can be supplied by saliva may also be an important factor in enamel remineralisation\(^26\). Laboratory experiments have indicated that, at equal degrees of supersaturation, an optimal rate of enamel remineralisation can be obtained with a calcium/phosphate ratio of 1.6\(^27\). However in plaque fluid and saliva, there is an excess of phosphate present, and the Ca/P ratio is approximately 0.3\(^10\). Therefore, it has been suggested that the calcium ion concentration may be the main rate-limiting mineral constituent\(^28\).

### Calcium delivery to plaque and saliva

A number of in vitro and clinical studies have investigated the delivery of additional calcium to plaque from oral care products and its potential benefits to oral health. For example, Pearce\(^31\) has demonstrated the ability to raise calcium concentrations in plaque using a mouthrinse containing urea and calcium chloride. Further, Pearce and Nelson\(^32\) have shown that such increased concentrations were associated with a reduction in softening and porosity of enamel surfaces covered with plaque in situ. Pearce et al.\(^33\) investigated a calcium and phosphate containing mouthrinse designed to precipitate HAP in plaque and showed that plaque fluid levels of calcium were elevated in subjects using the calcium/phosphate mouthrinse compared to a placebo mouthrinse following exposure to a sucrose solution.

Duke et al.\(^34\) investigated the addition of calcium glycerophosphate (CaGP) to a sodium monofluorophosphate (SMFP) containing calcium carbonate/silica toothpaste. Plaque collected from subjects using this toothpaste was shown to contain more calcium than plaque collected from subjects using the control toothpaste containing no CaGP. Similar results were found in approximal plaque collected after 1h following the use of CaGP containing toothpaste versus control toothpaste, and that the elevated levels were maintained for at least 8h after use\(^35\). In an in vitro biofilm flow cell model grown on bovine enamel, CaGP was pulsed in 1h before, during or 1h after a sucrose challenge and it was shown that there was a significant dose response of decreasing demineralisation as CaGP concentration increased, with pulsing before the sucrose challenge giving the greatest protection from demineralisation\(^36\). In a literature review on the role of CaGP and caries, the reduction of caries by CaGP containing toothpastes in a number of clinical trials is described and it was concluded that the elevation of calcium levels in plaque is the most likely explanation for the clinical effect\(^37\).

A dicalcium phosphate dihydrate (DCPD) containing toothpaste indicated an increase in the calcium activity and DS\(_{en}\) in plaque fluid 12h after use compared to a silica control\(^37,38\). Using \(^45\)Ca radiolabelled DCPD, it

---

Joiner et al.: Enhanced enamel benefits from a novel fluoride toothpaste
was shown that the calcium from the DCPD was incorporated into enamel with a concomitant reduction in enamel solubility in a rat model. In addition, an intra-oral brushing model with a fluoride/44Ca labelled DCPD toothpaste showed that 44Ca from DCPD could be detected in demineralised enamel after six days of treatment and in whole plaque 18h after the last treatment.

The incorporation of casein-phosphopeptide-stabilised amorphous calcium phosphate complexes (CPP-ACP) in a mouthwash have been demonstrated to increase the levels of calcium and phosphate ions in supragingival plaque, and to promote the remineralisation of enamel subsurface lesions in situ. It has been shown that the CPP-ACP binds to model plaque in vitro with twice the affinity of free calcium ions, providing a large calcium reservoir.

Chewing gums containing either monocalcium phosphate monohydrate or a mixture of dicalcium phosphate anhydrous and tetracalcium phosphate produced a very pronounced and persistent (16 minutes) elevation in the saturation of saliva with respect to tooth mineral. Following a sucrose rinse, the use of a chewing gum containing z-tricalcium phosphate significantly increased the calcium concentrations in saliva, plaque and plaque fluid, and increased the pH, compared to the use of a control chewing gum.

Laboratory experiments have shown that tooth mineral itself has the ability to buffer the pH and hence restrict the impact of cariogenic challenges. For example, Zaura et al. have shown that the pH at the bottom of in situ plaque formed in grooves cut of different materials and treated with glucose, was dependent on the solubility of the material, with dentine having a higher pH than enamel, which in turn was higher than polyacrylate. In another in vitro study where a dentine block was placed next to an enamel block, the dentine block acted as a sacrificial source of calcium and phosphate and inhibited the enamel dissolution in solutions initially undersaturated with respect to both minerals.

**Relationship between plaque calcium and fluoride**

There is considerable evidence in the literature that there is a linear relationship between levels of calcium and fluoride in plaque. It has been hypothesised that the ability of plaque to retain fluoride for longer periods is determined mainly by the calcium concentration in plaque. Further, it has been suggested that efforts to increase the cariostatic potential of fluoride could be based on methods to increase the concentration of calcium in plaque. To this end, Vogel et al. demonstrated that a calcium lactate pre-rinse before the use of a sodium fluoride mouthrinse significantly increased the one hour salivary fluoride concentration versus the sodium fluoride mouthrinse only. However, in a second experiment where a water rinse was used between the two rinses to reduce calcium carryover, salivary fluoride concentration was reduced, suggesting that the water rinse removed free calcium from oral tissues. The combination of the calcium lactate pre-rinse followed by a fluoride rinse also gave a significantly elevated overnight salivary fluoride concentration versus the fluoride rinse alone. The combination of a 150mM calcium lactate pre-rinse and a fluoride rinse was also shown to increase plaque and plaque fluid fluoride concentrations after one hour compared to the fluoride rinse alone. Elevated salivary fluoride levels after one hour were also obtained when using a 150mM calcium lactate pre-rinse, or a CaGP containing toothpaste followed by a sodium fluoride rinse, and when using the calcium lactate pre-rinse followed by a sodium fluoride toothpaste, suggesting that an increase in the concentration of calcium given shortly before a fluoride rinse or toothpaste may increase the cariostatic effect of the fluoride product. However, in contrast, when a 20mM calcium chloride pre-rinse was used in conjunction with a fluoride toothpaste, this combination had only a minor effect on salivary calcium and fluoride concentrations and none on plaque concentrations after 1 and 12 hours. In a further study with 150mM calcium lactate pre-rinse followed by a fluoride toothpaste, Pessan et al. confirmed significant elevated fluoride salivary concentrations and no significant elevation of plaque fluoride concentrations after 1h.

The elevation of calcium levels in plaque has the potential benefit of enhancing the remineralisation of enamel and aiding the potential of fluoride in this remineralisation process. For example, the remineralisation of artificially demineralised bovine enamel specimens was shown to be significantly improved by the use of a 150mM calcium lactate pre-rinse followed by the use of either a fluoride or non-fluoride toothpaste over 14 days in an in situ model. Blake-Haskins et al. demonstrated in vitro an increased remineralisation of subsurface enamel lesions with a combination of a calcium pre-rinse followed by a fluoride treatment versus fluoride treatment alone. The impact of calcium levels on enamel remineralisation is further confirmed by in vitro experiments using conditions found during a typical acid challenge in terms of pH, fluoride, calcium and phosphate concentrations, where it was found that significantly enhanced remineralisation of sub-surface enamel lesions was achieved by increasing the calcium concentration whilst maintaining fluoride concentration.

**Plaque-acid neutralisation and buffering by calcium carbonate**

It is known, from the classic studies of Stephan that following rinsing with a glucose solution, the pH of plaque can drop sharply and be maintained at a low pH for some considerable time due to the generation
of plaque acids. Further studies have demonstrated that this plaque pH drop is related to the susceptibility of an individual to be caries-prone or caries-free, with larger drops being exhibited by caries-prone subjects. Thus, routes that can limit the drop in plaque pH have the potential to reduce the impact of plaque acids on enamel demineralisation.

Calcium carbonate is an alkaline, buffering agent commonly used in toothpaste as an abrasive agent for cleaning and stain removal. Duke has described the deposition of calcium carbonate abrasive particles, with a mean diameter in the range 6-8 microns, into dental plaque for several hours after pre-treatment with a calcium carbonate-based toothpaste. The solubility of calcium carbonate particles increase in the presence of acid and have the potential to be partially dissolved when calcium carbonate particles increase in the presence of a calcium carbonate-based toothpaste. The solubility of dental plaque for several hours after pre-treatment with the deposition of calcium carbonate abrasive particles, will influence the concentration gradient within the local environment of the tooth surface and hence DS. Indeed, calcium carbonate-based toothpastes have been shown to reduce the pH drop of plaque using a number of in vitro and in vivo protocols.

The impact of calcium carbonate on enamel re- and demineralisation processes has been investigated using in situ protocols, where a calcium carbonate/SMFP toothpaste was shown to be significantly more effective at reducing enamel demineralisation and enhancing enamel remineralisation than a silica/SMFP toothpaste. In addition, it was recorded that more fluoride was found in the ‘test plaque’ treated with the calcium carbonate toothpaste. The authors conclude that these results suggest that calcium carbonate abrasive may enhance the effect of fluoride present in toothpaste on dental caries control.

Tooth erosion and calcium

Erosion is defined as the loss of hard tissue by chemical means not derived from bacteria, that is, the dissolution of hard tissue by acid where the acid source is not derived from oral bacteria. Erosion may be caused by either intrinsic sources, such as stomach acid reflux, or extrinsic sources, which are often associated with the consumption of acidic foods and beverages such as orange juice and cola. The calcium and phosphate contents of an acidic foodstuff or beverage are important factors for determining their erosive potential as they will influence the concentration gradient within the local environment of the tooth surface and hence DS. Indeed, the addition of calcium to acidic beverages such as fruit juice, soft drinks, carbonated beverages and sports drinks, and to acidic candies has been shown to significantly reduce their erosive potential.

**Calcium carbonate toothpaste containing micro-calcium**

Continuing efforts are being made by manufacturers to improve fluoride toothpaste by, for example, improving fluoride delivery/retention or by adding other beneficial agents. From the discussion above, the advantages of delivering a calcium source to the mouth which can give elevated calcium levels in the mouth has the potential to limit acid challenges by reducing enamel demineralisation whilst promoting enamel remineralisation. To this end, a combination of calcium sources (calcium glycerophosphate and micro-calcium carbonate, termed micro-calcium) was added to a calcium carbonate/SMFP toothpaste, in order to enhance the overall delivery of calcium to plaque and saliva with a concomitant improvement in tooth enamel benefits. This paper describes a series of in vitro and in vivo studies investigating the properties of this new toothpaste formulation including delivery of calcium to a biofilm, delivery of calcium to demineralised enamel and its impact on enamel hardness and the extent to which it can increase the concentration of calcium in saliva.

**Delivery of calcium to a biofilm in vitro**

**Materials and methods**

In order to evaluate the delivery of calcium from the new toothpaste containing micro-calcium to a plaque biofilm, the in vitro Calgary Biofilm Device (CBD) was used. In outline, the CBD allows a bacterial biofilm to be established on pegs attached to the lid of a 96-well plate. Biofilms can be treated and evaluated by incubation in 96-well plates containing various solutions. The Guggenheim consortium was used to represent the plaque biofilm and consisted of the following organisms: Streptococcus oralis, Streptococcus sobrinus, Actinomyces naeslundii, Fusobacterium nucleatum, Veillonella dispar and Candida albicans. The Guggenheim consortium was cultured as described by Shapiro et al. and then allowed to form a biofilm on whole human saliva pellicle coated pegs. The biofilms were treated for 1min with either the toothpaste containing micro-calcium, a commercially available calcium carbonate/SMFP toothpaste, a commercial silica-based toothpaste or water, with stirring at room temperature (n=16). The test toothpastes were prepared as slurries (1:3) in deionised sterile water. After product treatment, the pegs were washed three times with sterile water.

The QuantiChrom™ calcium kit (BioAssay Systems, CA, USA) was used to measure the amount of calcium delivered to the biofilm. For measurement of total calcium delivered to the plaque biofilm, the CBD (n=8) were treated with 1M hydrochloric acid for 60mins. An aliquot of the solution was removed (5µl) and to this was added the QuantiChrom™ reagent (200µl), followed by incubation for 10min and then reading absorbance at 550nm. The results were plotted as graphs showing calcium delivery.

---

Joiner et al.: Enhanced enamel benefits from a novel fluoride toothpaste
by 1M sodium hydroxide (5µl) and the absorbance read at 612nm. In order to measure the amount of soluble calcium present, the CBD (n=8) was incubated directly in the QuantiChrom™ reagent at room temperature. A standard curve was generated using known concentrations of calcium and this was used to calculate the soluble and total calcium concentrations.

**Results**

The total and soluble amounts of calcium measured in the *in vitro* biofilm following treatment with various toothpastes are shown in Table 1. Statistical analysis (ANOVA, Tukey-Kramer) showed that the biofilm treated with the toothpaste containing micro-calcium had a significantly (p<0.05) higher level of both soluble and total calcium when compared to the other three treatments. All other treatment comparisons were non significant for both soluble and total calcium.

**Calcium uptake by enamel from **$^{45}$Ca **radiolabelled micro-calcium carbonate in vitro**

**Materials and methods**

Paired bovine enamel blocks were cut using a diamond saw to give specimens of approximately 5x3mm. One set of the specimens was mounted in dental wax and the edges of the specimens painted using acid resistant nail varnish. Sub-surface lesions were created using an acidified 8% methyl cellulose gel (0.1M lactic acid, pH 4.6) with incubation at 37°C for 10 days. The specimens were analysed by Transverse Microradiography to ensure a balance in lesion depth and mineral loss. The other paired set of specimens was used as sound enamel. Four specimens were mounted on each perspex rod using dental wax and the edges were painted with the nail varnish. In total, there were eight sound and eight sub-surface lesions used in the experiment. The surface area of the specimens exposed to the toothpaste treatments was measured by optical methods.

A sample of the micro-calcium carbonate was $^{45}$Ca labelled using a neutron irradiation source. This isotope is a beta emitter with a half-life of 156 days. The radiolabelled micro-calcium carbonate was added to a 3:1 water:toothpaste slurry at an incorporation level of 2% w/w in the original toothpaste. An acidic buffer (50 mM acetic acid, 1.50 mM calcium chloride dihydrate, 0.90 mM potassium dihydrogen orthophosphate, 130 mM potassium chloride, pH 5.0) and neutral buffer (20 mM HEPES, 1.50 mM calcium chloride dihydrate, 0.90 mM potassium dihydrogen orthophosphate, 130 mM potassium chloride, pH 7.0) were also prepared. The bovine enamel specimens were cycled daily for 4 days through toothpaste slurry, acid buffer and neutral buffer with deionised water washings (15ml) between treatments according to Table 2. The specimens were then individually mounted onto a glass slide using dental wax. To each specimen was added 1M perchloric acid (50µl) for 3mins. This solution was removed with a pipette and placed into a labelled eppendorf tube. Each specimen was washed three times with 1M sodium acetate (50µl) and the washings combined with the acid treatment in the eppendorf tube. The perchloric acid and sodium acetate washings were repeated a further four times. The

### Table 1 Mean values of total and soluble calcium concentrations in an *in vitro* biofilm following various treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total [Ca] (s.d.) (mg/ml)</th>
<th>Soluble [Ca] (s.d.) (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-calcium toothpaste</td>
<td>3.196 (0.390)$^{a}$</td>
<td>0.041 (0.019)$^{a}$</td>
</tr>
<tr>
<td>Calcium carbonate toothpaste</td>
<td>0.273 (0.151)$^{b}$</td>
<td>0.006 (0.003)$^{b}$</td>
</tr>
<tr>
<td>Silica toothpaste</td>
<td>0.188 (0.186)$^{b}$</td>
<td>0.005 (0.002)$^{b}$</td>
</tr>
<tr>
<td>Water</td>
<td>0.024 (0.119)$^{b}$</td>
<td>0.001 (0.001)$^{b}$</td>
</tr>
</tbody>
</table>

Superscripts in columns with different letters are statistically different (p<0.05, Tukey Kramer)

### Table 2 pH cycling protocol

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early a.m.</td>
<td>Toothpaste slurry</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Acid buffer</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Neutral buffer</td>
<td>6-7 hours</td>
</tr>
<tr>
<td>Mid-late p.m.</td>
<td>Toothpaste slurry</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Acid buffer</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Neutral buffer</td>
<td>Overnight</td>
</tr>
</tbody>
</table>

### Table 3 $^{45}$Ca uptake to sound and subsurface lesion enamel specimens from a radiolabelled micro-calcium carbonate containing toothpaste (n=4)

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Mean Calcium Uptake (s.d.) (mg/mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sound enamel</td>
<td>3.15 (1.50)</td>
</tr>
<tr>
<td>Subsurface lesion</td>
<td>6.65 (1.07)</td>
</tr>
</tbody>
</table>
combined perchloric acid and sodium acetate washings were analysed for \(^{45}\)Ca and the mass of calcium uptake determined. This value was divided by the surface area of the specimen to give calcium uptake expressed as mg/mm\(^2\) and the mean calcium uptake values for sound and subsurface lesions calculated.

**Results**

The mean calcium uptake values for sound and subsurface lesions are shown in Table 3. Statistical analysis (t-Test, two sample two tailed) shows there to be a statistical difference (p<0.05) between the two types of specimens.

**Enamel remineralisation in vitro**

**Materials and methods**

Bovine enamel blocks (3x3mm) were treated with 1% citric acid, pH 3.8 for 20mins at room temperature. The surface microhardness (SMH) of the demineralised enamel blocks was then measured using a Mitutoyo HM122 Microhardness Tester (Mitutoyo Corp., Japan) and Knoop diamond under a load of 50g for 10s and stratified into three treatment groups (n=8). The enamel blocks were incubated with whole human saliva for 2h and rinsed with deionised water. They were then treated with either the micro-calcium containing toothpaste, a calcium carbonate/SMFP toothpaste or a non-fluoride silica-based toothpaste for 3mins. Toothpaste treatments were prepared as 1:2 (toothpaste:water) slurry in deionised water. The enamel blocks were then rinsed with deionised water for 30s and placed in a neutral buffer (pH 7.0) containing 20mM HEPES, 1mM CaCl\(_2\), 12.7mM KH\(_2\)PO\(_4\) and 130mM KCl, for 24 hours. After water rinsing, the SMH of the enamel blocks was re-measured and the change in SMH calculated.

**Results**

The change in SMH following various toothpaste treatments and a remineralising solution are shown in Table 4. Statistical analyses (ANOVA) showed an overall difference between treatments and paired comparisons (Tukey-Kramer) showed there were significant differences between all treatments (p<0.05).

**Evaluation of calcium delivery in vivo**

**Material and Methods**

Two double-blind, randomised, cross-over studies of identical design and study conditions were carried out. The objective of both studies was to compare total calcium concentration in saliva before and after brushing with toothpaste containing calcium carbonate abrasive plus micro-calcium (test) or brushing with toothpaste containing silica abrasive (control).

The study population (of each study) consisted of male and female adults who were of general good health. They had to have a minimum of 20 natural teeth, free of untreated caries or periodontal disease. Pregnant women and nursing mothers were excluded as were subjects with allergies to normal toothpaste ingredients.

At the start of each study subjects were required to give written informed consent, complete a medical history form, and undergo a dental screening to establish whether they were suitable. Subjects were given a calcium-free toothpaste to use throughout each study. In each study, subjects brushed their teeth at the study location for 1min with the test toothpaste (micro-calcium containing toothpaste) or the control toothpaste (silica abrasive). Both toothpastes contained fluoride either as 1.1% SMFP (test) or 0.32%NaF (control). Immediately before brushing and at fixed time intervals after brushing (5, 15, 30, 60min) unstimulated whole mouth saliva samples were taken. The total calcium concentration in the saliva samples was determined by an Inductively Coupled Plasma (ICP) Atomic Emission Spectrometer (Varian Vista ICP, Varian Instruments, Walnut Creek CA, USA). The protocols of both studies were reviewed and approved by an independent Research Ethics Committee.

The outcome variable was the total calcium concentration in saliva at each sampling time. In addition an integrated calcium delivery (ICD) value was determined. This was defined as the sum of the areas of the trapezoids formed by linear interpolation of neighbouring sampling times from 5-60min. The level of significance for all statistical analyses was set at p < 0.05.
Results

Study 1

A total of 37 subjects were enrolled and 36 subjects completed the study. The mean total calcium concentration (ppm) in saliva samples taken before (pre) and 15 min after brushing are shown in Table 5. The difference between the two groups in pre-brushing calcium concentrations was not statistically significant. In contrast, the calcium concentrations in saliva after brushing were significantly higher in the test group than in the control group (p<0.001).

The integrated calcium delivery (ICD) value between 5-60 min was 10,158 ppm*min for the test paste and 3,439 ppm*min for the control paste. The difference between the two groups was statistically significant (p<0.001). The ICD value in the test group was 5,583 ppm*min higher than the corresponding ICD value based on the normal, pre-brushing calcium concentration in saliva. This increase was statistically significant (p<0.001). In contrast, the ICD value in the control group was 1,071 ppm*min lower than the corresponding ICD value based on the normal, pre-brushing calcium concentration in saliva (p<0.001).

Study 2

A total of 38 subjects were enrolled and 37 subjects completed the study. The mean total calcium concentration (ppm) in saliva samples taken before (pre) and at 15 min after brushing are shown in Table 6. The difference between the two groups in pre-brushing calcium concentrations was not statistically significant. In contrast, the calcium concentrations in saliva after brushing were significantly higher in the test group than in the control group (p<0.001).

The integrated calcium delivery (ICD) value between 5-60 min was 10,562 ppm*min for the test paste and 3,224 ppm*min for the control paste. The difference between the two groups was statistically significant (p<0.001). The ICD value in the test group was 6,091 ppm*min higher than the corresponding ICD value based on the normal, pre-brushing calcium concentration in saliva. This increase was statistically significant (p<0.001). In contrast, the ICD value in the control group was 1,071 ppm*min lower than the corresponding ICD value based on the normal, pre-brushing calcium concentration in saliva (p<0.001).

Discussion

A number of in vitro biofilm models have been described in the literature for the evaluation of the effects of oral care products on various parameters such as biofilm removal and biocidal activity. The biofilm used in the current study is based on a consortium of orally relevant microbes and designed as a simple in vitro system for modelling supragingival plaque. The model has been further developed to test toothpaste formulations where it has been shown not to be significantly affected by major formulation components such as sodium lauryl sulphate. Further, the calcium delivery experiment in the current paper is a non-brushing protocol and so would tend to mimic delivery to a relatively undisturbed biofilm present in occlusal fissures, interproximal regions or other difficult to clean locations i.e. areas where caries incidence may be greater.

Under the conditions described in the current work, the in vitro biofilm had relatively low levels of total (acid extractable) and soluble calcium. Upon treatment with the silica or calcium carbonate toothpaste, there was a numerical increase in both total and soluble calcium but the difference from the water treatment was not of statistical significance. However, following treatment with the micro-calcium containing toothpaste, the total calcium increased by over eleven-fold and the free calcium by over six-fold, versus the calcium carbonate control toothpaste, demonstrating the delivery of additional calcium to the biofilm by the micro-calcium containing toothpaste. The significant increase in calcium concentration in the soluble form suggests that the micro-calcium containing toothpaste can increase the activity of calcium ions in plaque fluid, which might increase the driving force for remineralisation. Further, with the added delivery and retention of the calcium sources within plaque, this may give further protection by reducing plaque pH drop and/or acting as a calcium source to reduce undersaturation during a cariogenic challenge and serving as a new source of mineral ions between cariogenic challenges to promote remineralisation.

Nakashima et al. investigated a nano-sized calcium carbonate described as several tens to hundreds of nanometres in size. This was shown to exhibit better retention properties on oral surfaces than calcium carbonate abrasive particles of approximately 10 microns

International Dental Journal (2009) Vol. 59/No.4 (Supplement 1)
in diameter. Thus, the additional calcium delivery to the plaque from the micro-calcium containing toothpaste versus the calcium carbonate-based toothpaste in the current study is most likely due to the relative smaller particle size of the micro-calcium carbonate versus the regular calcium carbonate particles.

In the $^{45}$Ca labelled micro-calcium carbonate experiment pH cycling conditions were used as this approach has been described as one means of bringing in vitro models much closer to reality. Under these conditions, $^{45}$Ca was detected in both sound and subsurface lesion enamel specimens. This indicates that calcium ions from the micro-calcium carbonate has become available to be involved in the remineralisation process and is subsequently incorporated into the enamel specimens.

In the remineralisation study, the initial acid softening of the enamel with 1% citric acid (pH 3.8) is considered typical of the acid challenge from orange juice and has been used in a number of in vitro studies to evaluate erosion processes. The remineralisation solution in the current study was chosen to mimic concentrations found in plaque fluid, where the calcium:phosphate ratio is typically less than 0.5 i.e. relatively phosphate rich. Since caries is initiated and progressed beneath a layer of plaque, it therefore may be more important to study de- and remineralisation processes of enamel using experimental solutions representative of plaque fluid. Under such conditions, the micro-calcium containing toothpaste showed significantly more remineralisation than the calcium carbonate toothpaste or the non-fluoride toothpaste.

The two in vitro calcium delivery studies provided very consistent measures of total calcium concentration in saliva, both before and after brushing. Before brushing, the mean total saliva calcium concentrations were very similar in the test and control group in both studies ranging from 78-85ppm (equivalent to about 2mmol/l calcium ion).

These concentrations of calcium in saliva measured before brushing agree reasonable well with data reported in the literature. In this study baseline values were about 2mmol/l while data published by Sewon and co-workers showed 1.2-1.7mmol/l and Poureslami et al. found 1.4mmol/l. Normal ranges of 1-2 mmol/l are reported by Dawes in Edgar and O’Mullane.

After brushing with the test toothpaste, an increase in saliva calcium concentration was found in both studies which indicated delivery and deposition of calcium in the mouth. Again, very similar values were observed in the two studies, with at least a 2-fold increase in saliva calcium concentration measured 15min after brushing. In contrast, no increase in saliva calcium was apparent after brushing with the control toothpaste. In fact, 15min after brushing the calcium concentration in saliva was significantly lower than before brushing.

In addition to single time-point measurements of calcium concentration in saliva, an integrated measure of calcium delivery (ICD) was calculated, analogous to the methodology applied in pharmacokinetic studies and fluoride oral delivery studies. The results showed that the ICD value, calculated over 1h, was nearly three times higher after brushing with the test toothpaste than after brushing with the control toothpaste and twice as high compared to baseline.

**Conclusions**

Through the understanding of tooth enamel science and insights into the beneficial role calcium can play in the caries process a new fluoride toothpaste has been developed containing micro-calcium. It was shown in two clinical studies that more calcium was delivered to the mouth following the use of the micro-calcium containing toothpaste than compared to a silica toothpaste. The product differences were in excess of 50% and were of statistical significance (p<0.001).

An in vitro study has shown significantly (p<0.05) greater delivery of calcium to a plaque biofilm from the micro-calcium containing toothpaste than control toothpastes. A radiolabelled micro-calcium carbonate study has demonstrated the uptake of calcium to subsurface enamel lesions. In a remineralisation study, acid softened enamel became significantly harder (p<0.05) following treatment with the micro-calcium containing toothpaste than compared to a calcium carbonate toothpaste and non-fluoride silica toothpaste.

Therefore, the new toothpaste containing micro-calcium is able to deliver elevated levels of calcium to the mouth, promotes enhanced remineralisation of demineralised enamel lesions and thus can help repair early signs of tooth decay.

**References**


64. Brading MG, Cromwell VJ, Green AK et al. The role of triclosan in dentifrice formulations, with particular reference to a new 0.3% triclosan calcium carbonate-based system. Int Dent J 2004 54: 291-298.

Correspondence to: A. Joiner, Unilever Oral Care, Quarry Road East, Bebington, Wirral, CH63 3JW, UK. Email: Andrew.Joiner@Unilever.com