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Casein Phosphopeptide-Amorphous Calcium Phosphate Incorporated into Sugar Confections Inhibits the Progression of Enamel Subsurface Lesions in situ

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Key Words
Casein phosphopeptide-amorphous calcium phosphate · Enamel demineralization, prevention · Recaldent · Sugared confection

Abstract
Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been demonstrated to exhibit anticariogenic activity in randomized, controlled clinical trials of sugar-free gum and a tooth cream. Two randomized, double-blind, crossover studies were conducted to investigate the potential of CPP-ACP added to hard candy confections to slow the progression of enamel subsurface lesions in an in situ model. The confections studied were: (1) control sugar (65% sucrose + 33% glucose syrup); (2) control sugar-free; (3) sugar + 0.5% (w/w) CPP-ACP; (4) sugar + 1.0% (w/w) CPP-ACP; (5) sugar-free + 0.5% (w/w) CPP-ACP. Participants (10 and 14 in study 1 and 2) wore a removable palatal appliance containing enamel half-slabs with subsurface lesions, except for meals and oral hygiene procedures, and consumed 1 confection 6 times a day for 10 days. The enamel half-slabs were inset to allow the development of plaque on the enamel surface. Participants rested for 1 week before crossing over to another confection. The appliances were stored in a humid container at 37°C when not in the mouth. After each treatment period, the enamel half-slabs were removed, paired with their demineralized control half-slabs, embedded, sectioned and then analysed using transverse microradiography. In both studies consumption of the control sugar confection resulted in significant demineralization (progression) of the enamel subsurface lesions. However, consumption of the sugar confections containing CPP-ACP did not result in lesion progression, but in fact in significant remineralization (regression) of the lesions. Remineralization by consumption of the sugar + 1.0% CPP-ACP confection was significantly greater than that obtained with the sugar-free confection.
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Casein phosphopeptides (CPP), containing the cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu-, have a remarkable ability to stabilize amorphous calcium phosphate (ACP) in metastable solution [Cross et al., 2004, 2005]. Through the multiple phosphoseryl residues, the CPP bind to embryonic clusters of ACP preventing their growth to the critical size required for nucleation and phase transformation; hence, the calcium and phosphate ions of the ACP remain highly soluble and bio-available [Reynolds, 1998]. CPP-ACP complexes display anticariogenic activity in vitro, in animal models and in human

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in situ studies [Reynolds et al., 1995; Iijima et al., 2004; Oshiro et al., 2007; Rahiotis and Vougiouklakis, 2007; Cochrane et al., 2008].

The CPP-ACP complexes have been shown to reduce caries activity in specific-pathogen-free rats orally infected with *Streptococcus sobrinus* when CPP-ACP was applied twice daily in solution to the animals' molar teeth [Reynolds et al., 1995]. CPP-ACP has also been reported to significantly prevent enamel and dentine demineralization and promote remineralization in vitro [Reynolds, 1997; Oshiro et al., 2007; Rahiotis and Vougiouklakis, 2007]. Furthermore CPP-ACP added to various products such as sugar-free chewing gum, mouthwashes, milk and sugar-free confections significantly enhanced remineralization of enamel subsurface lesions in situ [Shen et al., 2001; Cai et al., 2003; Reynolds et al., 2003; Iijima et al., 2004; Walker et al., 2006; Cai et al., 2007]. Enamel subsurface lesions remineralized in situ by chewing sugar-free gum containing CPP-ACP were more acid resistant than normal tooth enamel [Iijima et al., 2004]. Significant remineralization of enamel subsurface lesions has also been observed in a human in situ study in which subjects consumed sugar-free pressed mint confections containing CPP-ACP 4 times a day for 14 days [Cai et al., 2003].

Recently, a randomized controlled clinical trial (RCT) demonstrated that chewing sugar-free gum containing CPP-ACP, relative to a normal sugar-free gum, reduced progression and enhanced regression of approximal caries in a population of schoolchildren in Australia [Morgan et al., 2008]. Other clinical RCTs have recently shown that CPP-ACP can regress white-spot lesions in post-orthodontic populations [Andersson et al., 2007; Bailey et al., 2009]. As CPP-ACP is an approved food ingredient, it may have the potential to be added to sugar confections to help lower their cariogenicity. The aim of this study was to investigate the effect of incorporating CPP-ACP into prototype sugar hard candy (boiled confections) on the progression and/or regression of enamel subsurface lesions in a clinical RCT utilizing an in situ model.

Materials and Methods

Study Design and Subject Recruitment

Two double-blind, randomized, crossover in situ studies were conducted to assess the capacity of CPP-ACP in sugar-containing hard candy (boiled confections) to prevent the progression of enamel subsurface lesions in situ. Approval for the studies was obtained from the University of Melbourne Human Research Ethics Committee. Healthy adult participants were recruited from staff and postgraduate students of the Melbourne Dental School, University of Melbourne. Ten participants (5 males and 5 females) aged 23–47 years were recruited for study No. 1, and 14 participants (8 males and 6 females) aged 21–47 years were recruited for study No. 2. Many of the participants were involved in both studies. The number of participants (10–14) required for the studies was based on our previous studies using the same in situ model and crossover design [Reynolds et al., 2008; Cai et al., 2009]. All participants had at least 22 natural teeth with no active caries, periodontal disease or other oral pathology, and none of them were using antibiotics or medications known to affect the salivary flow rate. Unstimulated saliva was collected for 2 min from subjects at rest whilst leaning forward with their heads tilted downwards, allowing saliva to flow into a preweighed centrifuge tube. Stimulated saliva was similarly collected for 2 min from subjects whilst they consumed each confection. All subjects had unstimulated and stimulated salivary flow rates in excess of 0.2 and 1.0 ml/min, respectively.

Intra-Oral Appliances

Removable mid-palatal acrylic appliances covering the palate from the first premolar to the last tooth in the arch were produced for each subject as described previously [Iijima et al., 2004]. The base of the appliance was retained in the mouth by 4 narrow-gauge stainless-steel circumferential clasps. The appliances were designed with bilateral troughs (15 mm long, 7 mm wide, 3 mm deep) cut into the base and designed to house the enamel slabs. The enamel slabs were retained by sticky wax as described by Iijima et al. [2004] to produce a 1-mm-deep trough above the enamel surface to allow plaque to establish and be retained.

Preparation of Enamel Subsurface Lesions

Subsurface enamel lesions were created using the technique employed by Iijima et al. [2004]. Sound relatively planar buccal and lingual surfaces free of cracks, stains and fluorotic lesions (as viewed under a dissecting microscope) were selected from extracted human third molars obtained from the Royal Dental Hospital of Melbourne and previously treated for at least 2 weeks in 10% (w/v) buffered formal-saline. The teeth were thrice rinsed with Milli-Q water, and the outer enamel surface was removed and polished wet to a mirror finish using Soflex (3M) discs on a slow-speed contra-angle dental handpiece. Each polished surface was then sawn from the tooth as a slab measuring approximately 8 × 4 mm, using a water-cooled diamond blade saw. The whole slab was then covered with acid-resistant nail varnish except for 2 (occlusal and gingival) mesiodistal windows as described by Iijima et al. [2004], each measuring 1 × 7 mm, separated from each other by 1 mm. Subsurface demineralized lesions were created in the enamel windows by immersing each slab in 40 ml unagitated demineralization buffer containing 20 g/l Carbopol 907 (carboxypolymethylene; BF Goodrich, Cleveland, Ohio, USA) [White, 1987], 500 mg/l hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif., USA), 0.1 mol/l lactic acid (Ajax Chemicals, Auburn, N.S.W., Australia), pH 4.8, for 4 days at 37°C. After 2 days, the slabs were removed from the buffer, rinsed thrice with Milli-Q water, blotted dry and placed into fresh demineralization buffer for another 2 days, then again rinsed and dried. After demineralization, each enamel slab was sawn through the midline of each window into two 4 × 4 mm half-slabs, and the cut surface of each half-slab was covered with nail varnish. One half-slab of each pair was retained as the demineralization control and stored in a labelled 1.7-ml microcentrifuge tube together with

a drop of Milli-Q water, to create a humidified environment. The other enamel half-slab of the pair was inset into an intra-oral appliance as described above with the subsurface enamel lesions exposed but recessed 1 mm below the surface of the appliance to create a plaque trap. Care was taken to keep the windows free of wax. Each two half-slab pairs were randomly assigned to either control or treatment. Four enamel half-slabs were inset into each appliance, two on each side in the bilateral troughs [Iijima et al., 2004].

Study Products and Randomization

For the two in situ studies, boiled confections (hard candy; 3.8 g each) were prepared by the Clinical and Consumer Group Cadbury-Adams Worldwide Research and Development (N.J., USA), some containing CPP-ACP (Recaldent CASRN 691364-49-5) obtained from Recaldent Pty Ltd. (Melbourne, Australia). For study 1, the 4 products tested were: (1) control sugar, (2) sugar + 0.5% w/w CPP-ACP, (3) sugar + 1.0% w/w CPP-ACP and (4) control sugar-free. For study 2, the 4 products tested were: (1) control sugar, (2) sugar-free + 0.5% w/w CPP-ACP, (3) sugar + 1.0% CPP-ACP and (4) control sugar-free. The confections were identical in appearance and taste and were provided as coded products in sealed packages and stored at room temperature. The sugar confections were 65% sucrose, 33% glucose syrup (corn syrup) and the remainder colouring and flavouring. The sugar-free confections were 95% w/w isomalt (palatinit) with the remainder colouring, flavouring and intense sweeteners (acesulfame K and aspartame).

For each study, the treatment sequences were generated by the Statistics and Analytical Sciences group of Cadbury-Adams Worldwide Research and Development and assigned to subject codes (fig. 1). Participants were then randomly assigned to subject codes by the study team (F.C. and P.S.). For each clinical study, the confection allocation was only divulged by the study sponsor after all data for that study had been acquired, data queries resolved and the database locked.

Study Protocols

Both studies were conducted at the Royal Dental Hospital of Melbourne, Melbourne, Australia, with subjects recruited and both studies completed within a 12-month period. Participants wore the appliances except during meals and oral hygiene procedures, and consumed 1 of the confections 6 times a day at 10.00 a.m., 11.00 a.m., 12.00 p.m., 2.00 p.m., 3.00 p.m. and 4.00 p.m., by allowing it to dissolve in the mouth, for 10 consecutive days (treatment period). The average time for the confection to dissolve was approximately 10 min. Following each treatment period, participants rested for 1 week (washout period), then crossed over to another confection. This was repeated until all 4 confection products for each study had been consumed by each participant. When the appliance was removed from the mouth, it was briefly rinsed with deionized water, kept in a sealed moist plastic bag and stored at 37°C. When wearing the appliance, participants were instructed not to eat anything except the study confections and not to drink anything except water. Participants were instructed to rinse and clean their appliances using a fluoride-free denture cleanser paste and toothbrush provided by the sponsor. They were also informed not to brush the area containing the enamel blocks. Participants kept a diary of confection consumption, brushing times and times the appliance was out of the mouth. At the beginning

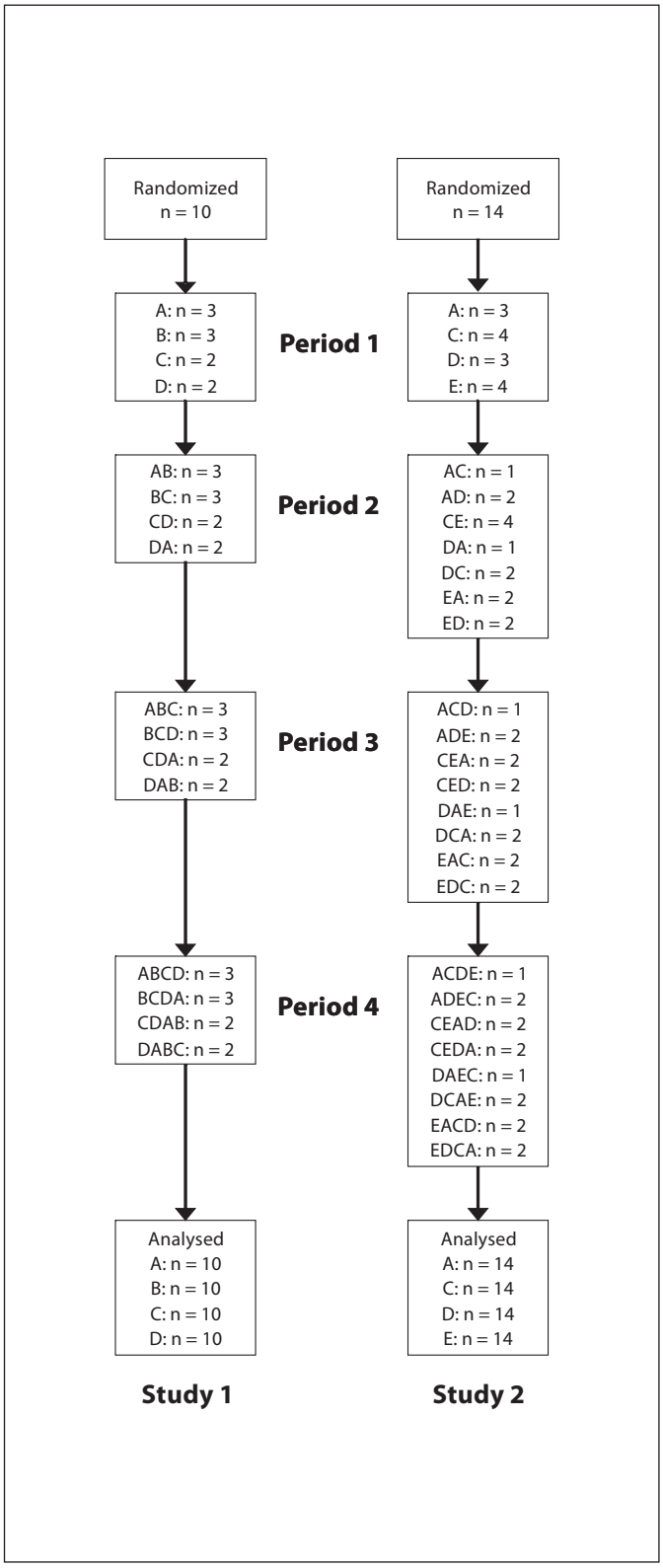

Fig. 1. Participant disposition for the two clinical studies. A = Sugar-free control; B = sugar + 0.5% CPP-ACP; C = sugar + 1.0% CPP-ACP; D = sugar control; E = sugar-free + 0.5% CPP-ACP.

and end of each day of each treatment period, participants brushed their teeth with a standard fluoride dentifrice (Colgate Cavity Protection) containing 1,000 μg/g F supplied by the sponsor and otherwise maintained their normal oral hygiene practices and dietary practices except for the restrictions during appliance wear times. After completion of each treatment period, the enamel slabs were visually inspected for plaque coverage and then removed from the appliances, rinsed with Milli-Q water and stored in moist, labelled microcentrifuge tubes.

Sectioning, Microradiography and Microdensitometric Image Analysis

After each treatment, the enamel half-slabs were paired with their respective control half-slabs and then dehydrated in absolute alcohol. Each pair of half-slabs was embedded, sectioned, lapped to 80 ± 5 μm and subjected to microradiography and computer-assisted densitometric analysis [Iijima et al., 2004]. Each section was radiographed along with an aluminium step wedge of increments 10 × 14 μm thick using Microchrome high-resolution glass plates (type 1A; Microchrome, USA) and nickel-filtered copper Kα radiation of 20 kV, 10 mA for 5 min. The radiographic apparatus used has been described previously by Malcolm [1972]. Radiographic images of the lesions were viewed using a Dialux 20 microscope (Leitz, Wetzlar, Germany). The images were acquired by a digital camera (Insight) using imaging software (Optimate version 5.2). Images of the lesions and the neighbouring areas of sound enamel were scanned using the programme's Ln line and Ln depth functions that give readings in grey values between 0 and 256 and the length of the scan, respectively. Each lesion was scanned 6 times through an area free of artefacts or cracks with each scan comprising 200 readings taken from the tooth surface through the lesion to sound enamel. An aluminium step wedge image on each slide was scanned and the averaged step grey value readings were plotted against aluminium thickness. The readings of the tooth section image within the linear portion of the step wedge curve and linear regression were used to convert the grey value data into values of equivalent thickness of aluminium. The section thickness was measured using a micrometre to ±5 μm and the mineral content (vol%) computed using the equation of Angmar et al. [1963] and the linear absorption coefficients of aluminium, organic matter plus water and apatitic mineral (131.5, 11.3 and 260.5, respectively). The image of the median strip of sound enamel between the two lesions was scanned 6 times and averaged to give a control densitometric profile of sound enamel. The lesion images (treated windows and demineralization control windows) to the gingival and occlusal side of the median strip of sound enamel were similarly scanned, as closely as possible to the median strip avoiding any irregularities commonly found at the lesion edges, and mineral content/depth profiles were computed.

Outcomes and Data Analysis

The mineral content/depth profile of each treated and control demineralized lesion was compared with the same profile of the median sound enamel in the same section. The difference between the areas under the densitometric profile of the demineralized control lesion and the median sound enamel, calculated by trapezoidal integration, is represented by ΔZ_c (integrated mineral loss). The difference between the areas under the densitometric profile of the treated lesion and the median sound enamel is represented by ΔZ_t. These ΔZ values were then used to calculate total mineral loss or gain ($\Delta Z_c - \Delta Z_t$) and percent mineral change (%MC), i.e. $\%MC = (\Delta Z_c - \Delta Z_t)/\Delta Z_c \times 100$. A negative $\Delta Z_c - \Delta Z_t$ or %MC value indicates mineral has been lost and therefore the lesion has progressed, whereas a positive value indicates mineral has been gained and therefore the lesion has regressed. The maximum mineral content of the surface layer (SL) and the minimum mineral content of the body of the lesion (BL) for each treated (t) and control (c) lesion were also measured and the percent change after treatment was calculated as $\%\Delta SL = (SL_t - SL_c)/SL_c \times 100$ and $\%\Delta BL = (BL_t - BL_c)/BL_c \times 100$. A negative %ΔSL or %ΔBL indicates there has been a loss of mineral from the SL or BL, respectively. A positive value indicates a gain in mineral content. Lesion depth (LD) was also measured for each lesion image and the percent change after treatment was calculated as %ΔLD. A negative %ΔLD indicates an increase in LD.

For each study, the primary outcome measure was total mineral loss or gain ($\Delta Z_c - \Delta Z_t$). This was compared across confection products using a repeated-measures analysis of variance (ANOVA) model with factors for treatment sequence, treatment period and confection type [Jones and Kenward, 2003]. The secondary outcome measures, SL_t, BL_t and LD_t were compared across confection products using repeated-measures ANOVA models with factors for treatment sequence, treatment period and confection type and covariate, SL_c, BL_c and LD_c, respectively. Repeated-measures ANOVA was also used to compare the control lesions between confection type. Post hoc comparisons of treatment differences were performed on the marginal means using the Sidak adjustment for multiple comparisons [SPSS, 2008]. ANOVA assumptions were checked using residual and normal probability plots. The unit of analysis was the participant. The outcome measures were determined for the treatment and control lesions for each enamel slab, and the resultant values averaged by participant and treatment period. p values less than 0.05 were regarded as being statistically significant. All analyses were conducted using either SPSS (version 17, SPSS Inc., Chicago, Ill., USA) or Stata (version 10, Stata Corp LP, College Station, Tex., USA) statistical software.

Results

All randomized participants completed both in situ studies with 100% reported compliance (fig. 1). No adverse events were reported in either study. The mean values for the enamel subsurface lesion parameters after exposure to the different confections in situ and their relative change compared with their original (control) lesions for both studies are presented in table 1. Analysis of the control lesions showed no statistically significant differences between confection types for any of the lesion parameters ΔZ_c, SL_c or BL_c. For the participants of both studies, their mean unstimulated salivary flow rate was 1.17 ± 0.71 ml/min, and their mean confection-stimulated salivary flow rate was 4.25 ± 1.78 ml/min. There was no significant difference in stimulation of salivary

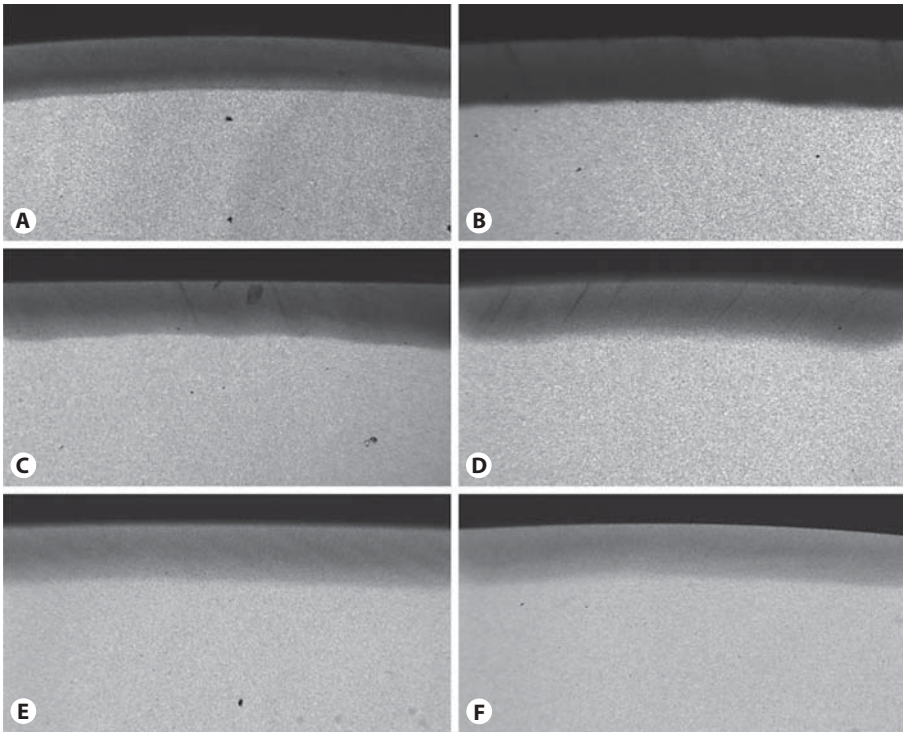

Fig. 2. Representative microradiographic images of enamel sections after consumption of the 5 confections in situ. **A** Representative image of lesions before treatment. **B** Representative image of lesions treated with control sugar confections. **C** Representative image of lesions treated with sugar confections containing 0.5% CPP-ACP. **D** Representative image of lesions treated with the control sugar-free confections. **E** Representative image of lesions treated with the sugar confections containing 1.0% w/w CPP-ACP. **F** Representative image of lesions treated with sugar-free confections containing 0.5% CPP-ACP.

flow rate by any of the confections used for the two studies.

Plaque developed in the troughs above the enamel half-slabs in the appliances by the end of each treatment period, such that it completely covered the surface of the enamel. In both studies consumption of the sugar control confection over the treatment period resulted in progression of the enamel subsurface lesions as shown by a significant decrease in total mineral content ($\Delta Z_c - \Delta Z_t$), an increase in LD and significant decreases in mineral content of the SL and of the BL (table 1). In contrast to the sugar control confections, the sugar confections containing CPP-ACP did not result in progression of the enamel subsurface lesions in either study (table 1). Consumption of the sugar + 0.5% CPP-ACP and sugar + 1.0% CPP-ACP confections did not result in a significant increase in LD (table 1) and in fact produced a significant increase in total mineral content and a significant increase in mineral content of the SL and the BL (table 1). Consumption of the sugar + 0.5% CPP-ACP and sugar + 1.0% CPP-ACP confections exhibited a clear CPP-ACP dose-related remineralization (regression) of the original enamel subsurface lesions. The increase in total mineral content produced by the sugar + 1.0% CPP-ACP confection was significantly greater than that produced by the sugar-free confection in both studies (table 1). The effects of the sugar in progressing the enamel subsurface lesions and the role of the CPP-ACP in negating the effects of the sugar and remineralizing (regressing) the subsurface lesions can be clearly seen in representative microradiographs of the lesions (fig. 2).

Discussion

Dental caries is a dynamic process involving periods of demineralization and remineralization. When demineralization is greater than remineralization, the lesion progresses. An in situ model used in this study allowed plaque to form over an enamel window already containing a defined and uniform subsurface demineralized lesion. Frequent consumption (6 times per day) of sugar confections using this model resulted in significant progression of the subsurface enamel lesions in the 10-day period confirming the cariogenicity of the confections. The same sugar confections with 0.5% (w/w) or 1.0% (w/w) CPP-ACP added did not result in lesion progression but in fact produced significant remineralization (regression) of the lesions in the same time period. The progression of the subsurface lesions following exposure

Table 1. Effect of CPP-ACP on enamel subsurface lesion parameters (means ± SD)

Confection type	Total mineral change $\Delta Z_c - \Delta Z_t$ (%MC) vol%min · μm	Surface layer SL_t (%ΔSL) vol%min	Body of lesion BL_t (%ΔBL) vol%min	Lesion depth LD_t (%ΔLD) μm
Study 1 (n = 10)				
Sugar control	–209.6 ± 65.3 (–6.1)	48.0 ± 9.1 (–12.7)	35.3 ± 11.6 (–18.5)	108.8 ± 3.3 (–11.6)
Sugar + 0.5% CPP-ACP	118.5 ± 39.0 (3.5)[a]	63.9 ± 4.0 (15.9)[a]	51.8 ± 3.8 (14.7)[c]	101.9 ± 3.0 (NC)[a]
Sugar-free control	150.4 ± 36.2 (4.8)[a]	65.1 ± 8.7 (9.1)[a]	52.0 ± 9.2 (14.4)[c]	97.5 ± 3.9 (NC)[c]
Sugar + 1.0% CPP-ACP	318.1 ± 74.4 (8.6)[a, b]	68.7 ± 5.8 (11.1)[a]	55.0 ± 6.9 (14.6)[a]	99.0 ± 1.9 (NC)[a]
	p < 0.0001	p = 0.001	p = 0.002	p = 0.0001
Study 2 (n = 14)				
Sugar control	–228.3 ± 128.7 (–7.5)	51.3 ± 4.9 (–7.0)	41.4 ± 6.4 (–10.4)	104.9 ± 5.1 (–7.6)
Sugar-free control	163.1 ± 54.6 (4.9)[a]	65.0 ± 10.3 (13.4)[a]	52.2 ± 12.6 (12.0)[c]	96.8 ± 7.9 (NC)[a]
Sugar + 1.0% CPP-ACP	357.1 ± 90.8 (11.0)[a, b]	70.7 ± 5.9 (20.9)[a]	57.9 ± 4.9 (25.1)[a, b]	99.6 ± 6.6 (NC)[a]
Sugar-free + 0.5% CPP-ACP	414.9 ± 80.5 (12.7)[a, b]	68.9 ± 8.8 (20.8)[a]	56.4 ± 9.7 (27.6)[a, d]	98.8 ± 3.8 (NC)[a]
	p < 0.0001	p < 0.0001	p < 0.0001	p = 0.0001

Figures in parentheses are percentages.

Total mineral change analysed using repeated-measures ANOVA models which included factors for treatment sequence, treatment period and confection type.

Surface layer analysed using repeated-measures ANOVA models which included factors for treatment sequence, treatment period and confection type, and a covariate SL_c; overall and multiple-comparisons test of confection type performed on marginal means for SL_t (evaluated at SL_c = 58.3 in study 1 and SL_c = 58.1 in study 2).

Body of lesion analysed using repeated-measures ANOVA models which included factors for treatment sequence, treatment period and confection type, and a covariate BL_c; overall and multiple-comparisons test of confection type performed on marginal means for BL_t (evaluated at BL_c = 47.0 in study 1 and BL_c = 46.8 in study 2).

Lesion depth analysed using repeated-measures ANOVA models which included factors for treatment sequence, treatment period and confection type, and a covariate LD_c; overall and multiple-comparisons test of confection type performed on marginal means for LD_t (evaluated aT LD_c = 98.0 μm in study 1 and 2).

NC = No change in lesion depth.

[a] $p \leq 0.005$: significantly different from sugar control after Sidak adjustment for multiple comparisons; [b] $p \leq 0.005$: significantly different from sugar-free control after Sidak adjustment for multiple comparisons; [c] $p \leq 0.05$: significantly different from sugar control after Sidak adjustment for multiple comparisons; [d] $p \leq 0.05$: significantly different from sugar-free control after Sidak adjustment for multiple comparisons.

to the sugar control confections is consistent with the sugar being metabolized by the plaque bacteria, present on the enamel slabs, to acid which then enhanced enamel subsurface demineralization (fig. 2). The presence of dental plaque on the enamel slabs was clearly visible, and significant subsurface enamel demineralization in 10-day exposure to the sugar control confections confirmed the plaque's viability and cariogenicity. The study was designed to encourage growth and maintenance of a viable plaque on the enamel lesions by appliance design and long intra-oral exposure times. Furthermore, when the appliances were out of the mouth, they were kept at 37°C in a humid environment to maintain plaque viability. Subjects were also instructed not to brush the enamel slabs on the appliance or eat anything but the confections when wearing the appliance.

CPP-ACP when incorporated into the sugar confections not only prevented the lesion progression observed with the control sugar confections, but also managed to remineralize (regress) the subsurface lesions that were initially present (fig. 2; table 1). The level of net demineralization that occurred with the control sugar confections highlights the ability of CPP-ACP to remineralize subsurface lesions notwithstanding the concomitant exposure to sugar and consequent acid challenge. Interestingly, Cochrane et al. [2008] have shown that CPP-ACP solutions could still produce net remineralization of enamel subsurface lesions in vitro even at pH 5.5. It was hypothesized that the slightly acidic environment helped to maintain the porosity of the lesion SL to allow the calcium and phosphate ions from the CPP-ACP to diffuse into the subsurface lesion. However, in that study remin-

eralization declined rapidly below pH 5.5 [Cochrane et al., 2008], and this would explain why the sugar confection containing 0.5% CPP-ACP did not remineralize as well as the sugar-free confection containing CPP-ACP at the same level. The efficacy of CPP-ACP in the sugar confections may also be partly explained by the capacity of CPP-ACP to bind to plaque as demonstrated by immunohistochemical staining of supragingival plaque in vivo [Reynolds et al., 2003]. CPP-ACP bound to the plaque overlying the enamel lesions would localize calcium and phosphate ions to help prevent demineralization and promote remineralization of the underlying lesion.

Consumption of the sugar-free control confection without CPP-ACP resulted in a small but significant level of remineralization through the natural remineralizing ability of saliva. Consumption of the sugar-free confection containing 0.5% (w/w) CPP-ACP resulted in a significantly higher level of remineralization than the sugar-free control. This is consistent with the already well-demonstrated efficacy of CPP-ACP as a remineralizing agent in sugar-free products including confectionery [Cai et al., 2003] and other sugar-free products such as chewing gum, mouthwashes and dentifrices in a variety of in situ studies [Shen et al., 2001; Reynolds et al., 2003; Iijima et al., 2004; Walker et al., 2006; Cai et al., 2007; Reynolds et al., 2008]. Recently these in situ studies were criticized because the stability of the mineral deposited in the subsurface lesions to acid challenge was not established [Zero, 2009]. However, in 3 of the published in situ studies, the remineralized enamel pieces were acid challenged in vitro after treatment to determine the stability of the mineral deposited in the lesions. These substantial posttreatment acid challenges showed that the enamel subsurface lesions remineralized with CPP-ACP were more acid resistant than normal tooth enamel [Iijima et al., 2004; Cai et al., 2007; Reynolds et al., 2008]. The increased acid resistance was attributed to the replacement of carbonated apatite with more acid-resistant apatite and also to the inhibition of enamel demineralization by adsorbed CPP-ACP. Zero [2009] has also criticized these studies stating that this posttreatment acid challenge, although substantive, is not equivalent to the demineralization/remineralization cycling conditions of the human mouth. He further criticized the in situ model used in the studies stating that in his opinion it was not conducive to oral biofilm formation [Zero, 2009]. The current study goes some way to address these criticisms by the frequent consumption (6 times per day) of sugar which produced a frequent acid challenge in the mouth and by the demonstration of the effects of a cariogenic biofilm that formed on the protected surface above the enamel pieces inset into the removable appliances. The incorporation of these changes into the in situ model (i.e. frequent sugar consumption and cariogenic biofilm development) produced results that confirmed those published previously showing net remineralization by the CPP-ACP after posttreatment in vitro acid challenge [Iijima et al., 2004; Cai et al., 2007; Reynolds et al., 2008].

The aim of any in situ model is to predict the outcome of a larger, clinical RCT whilst minimizing the risk to the subjects, controlling as many of the product-unrelated variables as possible and being mindful of subject comfort and compliance. The ultimate test of any in situ model is therefore its ability to predict the outcome of an RCT. As stated above, many published in situ studies on the CPP-ACP technology have demonstrated a significant remineralization of enamel subsurface lesions predicting a slowing of caries progression and promotion of lesion regression in an RCT. This prediction has now been validated in 4 published RCTs [Andersson et al., 2007; Morgan et al., 2008; Bailey et al., 2009; Rao et al., 2009]. One RCT demonstrated that CPP-ACP in a sugar-free chewing gum, relative to a normal sugar-free gum, significantly slowed progression and enhanced regression of approximal caries in a population of schoolchildren [Morgan et al., 2008]. Another 2 RCTs demonstrated that CPP-ACP delivered in a tooth cream significantly regressed white-spot lesions in postorthodontic populations [Andersson et al., 2007; Bailey et al., 2009], and recently another RCT showed that CPP and calcium carbonate in a toothpaste formulation, which would spontaneously form CPP-ACP in saliva, significantly reduced caries experience in children compared with a placebo toothpaste [Rao et al., 2009].

In conclusion, this in situ study showed that consumption of sugar confections containing CPP-ACP significantly remineralized (regressed) subsurface enamel lesions. The results suggest that incorporation of CPP-ACP into sugar confections may reduce their cariogenicity.

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References

Andersson A, Skold-Larsson K, Hallgren A, Petersson LG, Twetman S: Effect of a dental cream containing amorphous calcium phosphate complexes on white spot lesion regression assessed by laser fluorescence. Oral Health Prev Dent 2007;5:229–233.

Angmar B, Carlstrom D, Glas J: Studies on the ultrastructure of dental enamel. J Ultrastruct Res 1963;8:12–23.

Bailey DL, Adams GG, Tsao CE, Hyslop A, Escobar K, Manton DJ, Reynolds EC, Morgan MV: Regression of post-orthodontic lesions by a remineralizing cream. J Dent Res 2009; 88:1148–1153.

Cai F, Manton DJ, Shen P, Walker GD, Cross KJ, Yuan Y, Reynolds C, Reynolds EC: Effect of addition of citric acid and casein phosphopeptide-amorphous calcium phosphate to a sugar-free chewing gum on enamel remineralization in situ. Caries Res 2007;41:377–383.

Cai F, Shen P, Morgan MV, Reynolds EC: Remineralization of enamel subsurface lesions in situ by sugar-free lozenges containing casein phosphopeptide-amorphous calcium phosphate. Aust Dent J 2003;48:240–243.

Cai F, Shen P, Walker GD, Reynolds C, Yuan Y, Reynolds EC: Remineralization of enamel subsurface lesions by chewing gum with added calcium. J Dent 2009;37:763–768.

Cochrane NJ, Saranathan S, Cai F, Cross KJ, Reynolds EC: Enamel subsurface lesion remineralisation with casein phosphopeptide stabilised solutions of calcium, phosphate and fluoride. Caries Res 2008;42:88–97.

Cross KJ, Huq NL, Palamara JE, Perich JW, Reynolds EC: Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. J Biol Chem 2005;280:15362–15369.

Cross KJ, Huq NL, Stanton DP, Sum M, Reynolds EC: NMR studies of a novel calcium, phosphate and fluoride delivery vehicle-alpha (S1)-casein(59–79) by stabilized amorphous calcium fluoride phosphate nanocomplexes. Biomaterials 2004;25:5061–5069.

Iijima Y, Cai F, Shen P, Walker G, Reynolds C, Reynolds EC: Acid resistance of enamel subsurface lesions remineralized by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. Caries Res 2004;38:551–556.

Jones B, Kenward MG: Design and Analysis of Crossover Trials, ed 2. Boca Raton, Chapman & Hall/CRC, 2003.

Malcolm AS: A microradiographic apparatus. Aust Dent J 1972;17:454–460.

Morgan MV, Adams GG, Bailey DL, Tsao CE, Fischman SL, Reynolds EC: The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. Caries Res 2008;42:171–184.

Oshiro M, Yamaguchi K, Takamizawa T, Inage H, Watanabe T, Irokawa A, Ando S, Miyazaki M: Effect of CPP-ACP paste on tooth mineralization: an FE-SEM study. J Oral Sci 2007;49:115–120.

Rahiotis C, Vougiouklakis G: Effect of a CPP-ACP agent on the demineralization and remineralization of dentine in vitro. J Dent 2007;35:695–698.

Rao SK, Bhat GS, Aradhya S, Devi A, Bhat M: Study of the efficacy of toothpaste containing casein phosphopeptide in the prevention of dental caries: a randomized controlled trial in 12- to 15-year-old high caries risk children in Bangalore, India. Caries Res 2009;43: 430–435.

Reynolds EC: Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. J Dent Res 1997;76:1587–1595.

Reynolds EC: Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: a review. Spec Care Dentist 1998;18:8–16.

Reynolds EC, Cai F, Cochrane NJ, Shen P, Walker GD, Morgan MV, Reynolds C: Fluoride and casein phosphopeptide-amorphous calcium phosphate. J Dent Res 2008;87:344–348.

Reynolds EC, Cai F, Shen P, Walker GD: Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. J Dent Res 2003;82:206–211.

Reynolds EC, Cain CJ, Webber FL, Black CL, Riley PF, Johnson IH, Perich JW: Anticariogenicity of calcium phosphate complexes of tryptic casein phosphopeptides in the rat. J Dent Res 1995;74:1272–1279.

Shen P, Cai F, Nowicki A, Vincent J, Reynolds EC: Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. J Dent Res 2001;80:2066–2070.

SPSS: Statistics Base 17.0 User's Guide. Chicago, SPSS Inc, 2008.

Walker G, Cai F, Shen P, Reynolds C, Ward B, Fone C, Honda S, Koganei M, Oda M, Reynolds EC: Increased remineralization of tooth enamel by milk containing added casein phosphopeptide-amorphous calcium phosphate. J Dairy Res 2006;73:74–78.

White DJ: Use of synthetic polymer gels for artificial carious lesion preparation. Caries Res 1987;21:228–242.

Zero DT: Recaldent – evidence for clinical activity. Adv Dent Res 2009;21:30–34.