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Acid Resistance of Enamel Subsurface Lesions Remineralized by a Sugar-Free Chewing Gum Containing Casein Phosphopeptide-Amorphous Calcium Phosphate

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Key Words
Casein phosphopeptide-stabilized amorphous calcium phosphate · Enamel subsurface lesion remineralization · Acid resistance

Abstract
The aim of this clinical study was to investigate the acid resistance of enamel lesions remineralized in situ by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP: Recaldent™). The study utilized a double-blind, randomized, crossover design with two treatments: (i) sugar-free gum containing 18.8 mg of CPP-ACP, and (ii) sugar-free gum not containing CPP-ACP as control. Subjects wore removable palatal appliances with insets of human enamel containing demineralized subsurface lesions and chewed the gum for 20 min 4 times per day for 14 days. After each treatment the enamel slabs were removed and half of each lesion challenged with acid in vitro for 8 or 16 h. The level of remineralization was determined using microradiography. The gum containing CPP-ACP produced approximately twice the level of remineralization as the control sugar-free gum. The 8- and 16-hour acid challenge of the lesions remineralized with the control gum resulted in 65.4 and 88.0% reductions, respectively, of deposited mineral, while for the CPP-ACP-remineralized lesions the corresponding reductions were 30.5 and 41.8%. The acid challenge after in situ remineralization for both control and CPP-ACP-treated lesions resulted in demineralization underneath the remineralized zone, indicating that the remineralized mineral was more resistant to subsequent acid challenge. The results show that sugar-free gum containing CPP-ACP is superior to an equivalent gum not containing CPP-ACP in remineralization of enamel subsurface lesions in situ with mineral that is more resistant to subsequent acid challenge.

Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) have been demonstrated to have anticariogenic activity in laboratory, animal and human in situ experiments [Reynolds, 1998; Reynolds et al., 1999, 2003; Shen et al., 2001]. 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. Through the multiple phosphoseryl residues, the CPP bind to forming nanoclusters of ACP, preventing their growth to the critical size required for nucleation and phase transformation [Reynolds, 1998].

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The CPP-ACP nanocomplexes have been shown to reduce caries activity in specific-pathogen-free rats orally infected with *Streptococcus sobrinus* when applied as a solution to their molar teeth [Reynolds et al., 1995]. The CPP-ACP significantly reduced smooth-surface caries activity in a dose-response fashion with 0.1% w/v CPP-ACP producing a 14% reduction and 1% w/v CPP-ACP a 55% reduction relative to the distilled water control. In a human in situ enamel demineralization study, a 1% w/v CPP-ACP solution used twice daily produced a 51 ± 19% reduction in enamel mineral loss caused by frequent sugar-solution exposure [Reynolds, 1998]. The CPP-ACP solutions have also been shown to significantly remineralize enamel subsurface lesions in vitro where a 1.0% w/v CPP-ACP solution replaced 64 ± 20% of mineral lost [Reynolds, 1997]. CPP-ACP in sugar-free chewing gum have recently been shown to significantly remineralize enamel subsurface lesions in situ [Shen et al., 2001]. This study showed that gum containing 56.4 mg of CPP-ACP increased enamel subsurface remineralization by 159% relative to a control gum not containing CPP-ACP. In a recent mouthwash study, CPP-ACP was shown localized in plaque where it significantly increased the contents of calcium and inorganic phosphate [Reynolds et al., 2003].

It is of interest to determine the resistance of the enamel subsurface lesions remineralized by CPP-ACP to subsequent acid challenge, as remineralized enamel may be poorly structured and therefore more susceptible to acid challenge than normal enamel. The anticaries effect of CPP-ACP would be significantly enhanced if the mineral it promotes is relatively more resistant to further acid challenge than normal tooth enamel. The aim of this study therefore was to investigate the acid resistance of enamel subsurface lesions remineralized in situ by a sugar-free chewing gum containing CPP-ACP (Recaldent™).

Materials and Methods

Study Design and Subject Recruitment

This study was a double-blind, randomized, crossover design and approval was obtained from The University of Melbourne Human Research Ethics Committee and the Royal Dental Hospital of Melbourne Ethics in Clinical Research Committee. Ten healthy adult subjects (5 males and 5 females) were recruited from the staff and postgraduate students (age 21–45 years) of the School of Dental Science. An intraoral examination confirmed that each had at least 22 natural teeth with no current caries activity, periodontal disease or other oral pathology. None of the subjects were using antibiotics or medications, which could have affected salivary flow rate. Unstimulated salivary flow rates were measured by instructing the subjects to lean forward with their heads tilted downward allowing saliva to flow into a preweighed 15-ml centrifuge tube for 2 min. Stimulated salivary flow rates were measured in the same manner while the subjects chewed sugar-free gum.

Preparation of Intraoral Appliances and Enamel Subsurface Lesions

Removable mid-palatal acrylic appliances covering the first premolars to the last tooth in the arch were produced for each subject as described previously [Shen et al., 2001]. Extracted human third molars were obtained from the Royal Dental Hospital of Melbourne and prepared as described previously [Shen et al., 2001]. The outer enamel surface was polished wet to a mirror finish using Soflex™ (3M, St. Paul, Minn., USA) disks on a slow-speed contra-angle dental handpiece. Each polished surface was then sawn from the tooth as an approximately 10 × 4 mm slab, using a water-cooled diamond blade saw and the whole slab covered with acid-resistant nail varnish except for two (occlusal and gingival) mesiodistal windows (approximately 9 × 1 mm) separated from each other by 1 mm (fig. 1).

Lesions were created in the enamel windows using the demineralization buffer of White [1987] containing 20 g/l Carbopol 907 (carboxypolymethylene; BF Goodrich, Cleveland, Ohio, USA), 500 mg/l hydroxyapatite (Bio-Gel, HTP; Bio-Rad Laboratories, Richmond, Calif., USA) and 0.1 mol/l lactic acid (Ajax Chemicals, Auburn, N.S.W., Australia), pH 4.8 for 16 h at 37°C. Each enamel slab was placed in 40 ml of demineralization buffer. After demineralization each enamel slab was sawn perpendicular to the windows into 3 × 4 mm^2 (one-third) and 6 × 4 mm^2 (two-third) blocks and the cut surfaces of each block covered with nail varnish (fig. 1). The one-third block was retained as the demineralization control and stored in a labeled 1.5-ml microcentrifuge tube together with a drop of Milli-Q water, thereby creating a humidified environment. The two-third enamel blocks were inset into bilateral troughs in each intraoral appliance and retained using dental wax for the remineralization protocol. Care was taken to keep the windows free of wax. Four enamel blocks (two-third blocks) were inset into each appliance, two on each side. The enamel blocks were changed for every test period. After each treatment period the treated enamel blocks were sectioned through the window midline and half of each window covered with nail varnish, leaving half the lesion windows exposed (fig. 1). The varnish-coated blocks were then subjected to further acid challenge in vitro by exposure to the pH 4.8 demineralization buffer described above (40 ml per block) for 8 or 16 h at 37°C.

Experimental Protocol

The sugar-free (xylitol) chewing gums were provided by Cadbury Adams Global, R&D (Morris Plains, N.J., USA) as coded products and stored in a secured area at room temperature. The gums were of the slab type and weighed approximately 2.5 g/slab. One of the gums contained 18.8 mg of CPP-ACP but was identical to the control gum in all other respects. The two treatments consisting of the two gum products were randomized into coded treatments 1 and 2. The study protocol was similar to that described previously [Shen et al., 2001] where each subject chewed the sugar-free gum for 20 min, 4 times a day for 14 days at the following times: 10.00 a.m., 11.30 a.m., 2.00 p.m., and 3.30 p.m. The intraoral appliance was not worn during consumption of food or drink (except the experimental gum) or oral hygiene procedures, and when the appliance was removed, it was stored in a sealed moist plastic bag at room temperature. Subjects were instructed to rinse and clean their appliances using deionized water. Following the 2-week testing period, there was a 1-week washout period. The subjects crossed over to each of the two treatments.

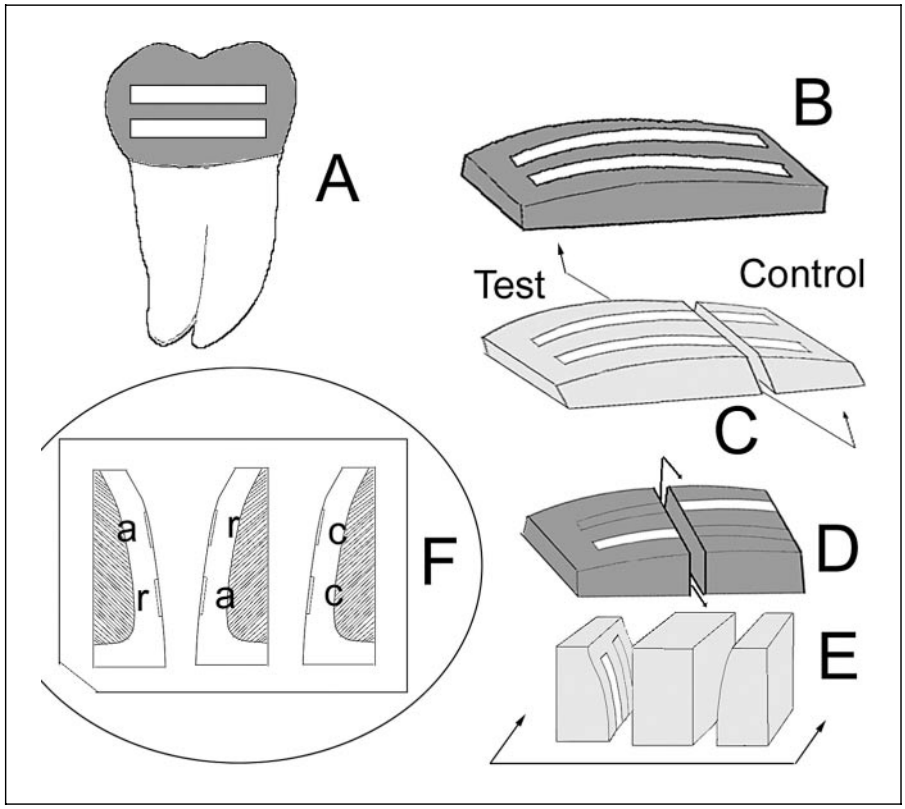

Fig. 1. Lesion preparation: extracted human third molars were polished and painted with nail varnish to form two mesiodistal windows (A). An enamel slab (B) was sawn from the painted aspect of the tooth. After demineralization, it was cut perpendicular to the windows into a test (two-third) and a control (one-third) block (C). After in situ remineralization, the test block was further cut into two through the midline of each window and painted with nail varnish leaving two half-windows exposed (D) for acid challenge. After the acid challenge the nail varnish was removed and the blocks were positioned with the windows parallel to each other (E), embedded and sectioned. Each section was marked to indicate the remineralized lesion (F, r), the acid-challenged lesion (F, a) and the control lesion (F, c).

Subjects kept a diary of gum use. No alterations were made to the subjects' diet and oral hygiene procedures for the duration of the study. All subjects lived in a city which had the reticulated water supply fluoridated at 1 ppm and used fluoride-containing toothpaste (1,000 ppm) after breakfast and before retiring at night.

Sectioning and Microradiography

Each demineralized control enamel block combined with the two treated blocks (fig. 1) were rinsed in acetone to remove the nail varnish. The blocks were then dehydrated in absolute alcohol and then placed into freshly poured, transparent, cold-curing methacrylate resin (Paladur; Heraeus Kulzer, Germany) with the lesion windows parallel (fig. 1). The resin vial was marked at the top corner to identify the test and control blocks, and the resin was allowed to set at room temperature overnight. Two transverse sections approximately 200 μm thick each were cut from the embedded blocks perpendicular to the lesion surface through the midline of the three blocks using an internal annulus saw microtome (Leica 1600; Leica, Germany) (fig. 1). The sections were lapped down to 80 ± 5 μm using a RotoPol-21/RotoForce4 lapping instrument (Struers, Denmark) with 1,200- and 2,400-grit lapping paper. The lapped sections were removed from the lapping instrument with absolute ethanol and rinsed in deionized water, blotted dry and stored on soft tissue paper between glass slides. Each section was radiographed along with an aluminium stepwedge of 10 × 14 μm-thick increments using Microchrome High Resolution glass plates (Type 1A; Microchrome, USA) and nickel-filtered copper Kα radiation at 20 kV, 10 mA for 5 min. The radiographic apparatus used has been described previously by Malcolm [1972]. Each glass plate was developed in 20 ml of Microchrome Developer D5 (1:4 dilution; Microchrome) for 4 min, placed into glacial acetic acid stop bath for 30 s and then fixed in Microchrome Fixer F4 (1:4 dilution; Microchrome) for 4 min. The temperature of all the photochemicals was maintained at 20°C by a water bath.

Radiographic images of the lesions were viewed via transmitted light through a Dialux 20 microscope (Ernst Leitz, Wetzlar, Germany). The images were acquired by a digital camera (Spot Insight; Diagnostic Instruments, Inc., Mich., USA) and analyzed by using imaging software Optimate version 5.2 running on a PC (Pentium III). Images of the lesions and the neighboring areas of sound enamel were scanned using the program's line luminance function that gives readings in gray values between 0 and 256. An area free of artifacts or cracks was selected for analysis. Each scan comprised 200 readings taken from the tooth surface through the lesion to sound enamel. The aluminium stepwedge image on each slide was scanned, and the averaged step gray value readings were plotted against aluminium thickness. Linear regression was used to convert the gray value data into values of equivalent thickness of aluminium. The section thickness was measured and the percent mineral data 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 sound-enamel densitometric profile. The lesion images (remineralization test windows with or without acid

Table 1. Remineralization of enamel sub-surface lesions by sugar-free chewing gum before and after acid challenge

	Demineralization		Remineralization								
			no acid challenge			8-hour acid challenge			16-hour acid challenge		
	Ld µm	ΔZd vol%min · µm	Ld µm	ΔZd – ΔZr vol%min · µm	%R	Ld µm	ΔZd – ΔZr vol%min · µm	%R	Ld µm	ΔZd – ΔZr vol%min · µm	%R
Control	87.2 ± 11.5[a,b]	3,363 ± 557[c]	86.0 ± 12.4[b]	303 ± 25	9.02 ± 0.74	88.7 ± 12.7[b]	105 ± 30	3.12 ± 0.88	89.0 ± 11.2[b]	36 ± 34	1.08 ± 1.02
CPP-ACP	88.3 ± 14.0[b]	3,719 ± 443[c]	87.3 ± 11.7[b]	665 ± 36	17.88 ± 0.97	87.6 ± 17.2[b]	462 ± 33	12.43 ± 0.90	91.0 ± 12.0[b]	387 ± 44	10.40 ± 1.19

[a] Mean ± standard deviation (n = 10).
[b,c] Values similarly marked were not significantly different ($p > 0.05$).

challenge and demineralized control windows) to the gingival and occlusal side of the median strip of sound enamel were similarly scanned, as close as possible to the median strip but avoiding any irregularities commonly found at the lesion edges, and the percent mineral profiles were computed.

Remineralization Data Analysis

The percent mineral profile of each enamel block's demineralized and remineralized lesion (including remineralized and then acid-challenged) was compared with that of the median sound enamel between the lesions of the same section. The difference between the areas under the densitometric profile of the demineralized lesion and the median sound enamel, calculated by trapezoidal integration, is represented by ΔZd. The difference between the areas under the densitometric profile of the remineralized lesion and the median sound enamel, calculated by trapezoidal integration, is represented by ΔZr. These parameters were then converted to percent change values after remineralization for each lesion, as such, percent remineralization (%R) represents the percent change in ΔZ values:

$$\%R = \frac{\Delta Zd - \Delta Zr}{\Delta Zd} \times 100.$$

Normality of the data was confirmed using normal probability plots and the Kolmogorov-Smirnov test. Homogeneity of variance was confirmed using Levene's test. The %R and lesion depth (Ld) data were analyzed using a two-way analysis of variance for a randomized complete block design with a post-hoc Scheffé test [Sokal and Rohlf, 1969]. All statistical analyses were performed using SPSS version 11.0 software.

Results

The mean age of the subjects was 32.3 ± 7.9 years and the mean unstimulated salivary flow rate was 0.51 ± 0.28 ml/min and the mean stimulated salivary flow was 2.13 ± 0.92 ml/min. On obtaining the complete data set, the randomization code was released and the data decoded. The original ΔZd and lesion depth values as well as the level of enamel remineralization before and after 8- and 16-hour acid challenge are presented in table 1. The result for each subject represents the average of 96 scans comprising 12 scans (6 each from the gingival and occlusal lesions) on each of the two sections from the four tooth blocks used per subject for each treatment. Consumption of the gum containing CPP-ACP produced 17.9 ± 1.0% enamel subsurface lesion remineralization compared with 9.0 ± 0.7% remineralization produced by the control sugar-free gum. The difference between these groups was statistically significant ($p < 0.05$). Acid challenge of the remineralized lesions for 8 and 16 h resulted in 65.4 and 88.0% reductions in enamel remineralization for those previously remineralized by the control sugar-free chewing gum (table 1). The corresponding reductions for the lesions remineralized with CPP-ACP sugar-free gum by the 8- and 16-hour acid challenge were 30.5 and 41.8%, respectively, such that the final mineral levels were significantly higher (4- and 10-fold, respectively) than those of the control gum lesions after 8- and 16-hour acid challenge (table 1). Representative microradiographs of the lesions before and after remineralization as well as after acid challenge for 8 and 16 h are presented in figure 2. The microradiographic images of the lesions revealed a similar pattern of demineralization for both the control and CPP-ACP-treated lesions upon subsequent acid challenge, where the bulk of the secondary demineralization tended to occur below the remineralized zone. Further, as the ΔZr values after the same acid challenge conditions as those that produced the original lesions were never greater than the original ΔZd values (that is ΔZd – ΔZr never became a negative value; table 1), then this indicates that the remineralized lesions were more resistant to subsequent acid challenge. Although the lesion depths tended to become smaller with remineralization and larger with secondary

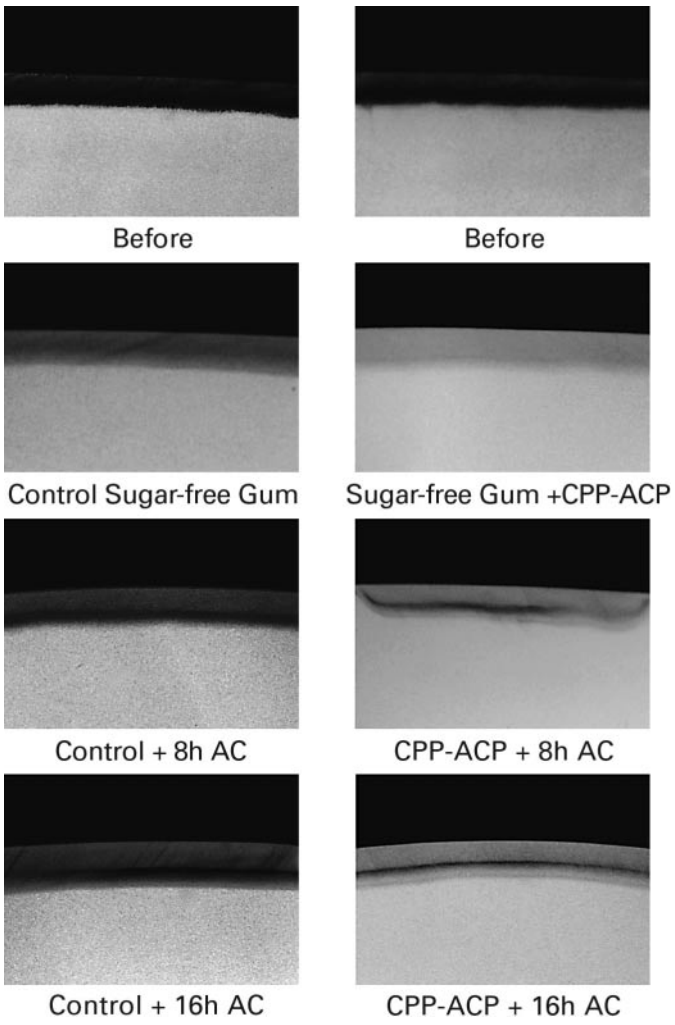

Fig. 2. Representative microradiographs of the enamel subsurface lesions before and after remineralization as well as after 8- and 16-hour acid challenge (AC).

acid challenge, the mean values for each group were not significantly different from the initial mean values (table 1). In fact, the control and CPP-ACP-treated mean lesion depths were not significantly different from each other for any condition (table 1).

Discussion

CPP have been shown not only to stabilize amorphous calcium phosphate, but also to deliver and localize ACP at the tooth surface [Reynolds, 1998; Reynolds et al., 1999]. A recent study [Reynolds et al., 2003] has demonstrated that CPP could still be detected on the tooth surface 3 h after consuming xylitol gum containing CPP-ACP. Other studies have demonstrated that CPP-ACP in a mouthwash significantly increased the level of calcium and inorganic phosphate ions in supragingival plaque with the CPP bound to salivary pellicle and to the surface of bacteria in the plaque biofilm [Reynolds, 1998; Reynolds et al., 1999, 2003]. The present study shows that 18.8 mg of CPP-ACP delivered in a sugar-free gum significantly remineralized enamel subsurface lesions in situ approximately 2 times better than a control sugar-free gum. These results therefore confirm those of Shen et al. [2001], who also showed that CPP-ACP remineralizes enamel subsurface lesions in situ. The present study, however, extends these results by showing that CPP-ACP produces significantly greater remineralization of enamel subsurface lesions even after 8- and 16-hour acid challenge. The representative microradiographs in figure 2 clearly show that CPP-ACP produced significantly greater remineralization throughout the body of the lesion, and this was still apparent after 16 h of acid challenge.

Interestingly, for both the control and CPP-ACP-treated lesions the pattern of demineralization after secondary acid challenge was similar, with the bulk of the demineralization tending to occur underneath the remineralized zone. This together with the amount of mineral lost indicates that the remineralized enamel was more resistant to acid challenge than the original enamel. Remineralized enamel may be more resistant than normal tooth enamel to acid challenge, as normal enamel is a calcium-deficient, carbonated apatite that has been found to be more soluble than hydroxyapatite, which has been attributed to differences in crystallinity and/or microstrain [Nelson, 1981; Le Geros et al., 1996; Baig et al., 1999; Shellis et al., 1999]. As CPP-ACP promotes remineralization of enamel subsurface lesions with hydroxyapatite [Reynolds, 1997], then in a relatively low carbonate environment of the subsurface lesion in situ the CPP-ACP-promoted mineral may have improved crystallinity and lower microstrain than normal tooth enamel, thereby helping to explain the results of this study. It should also be noted that the subjects in this study were using a fluoride-containing dentifrice twice a day such that any residual fluoride may have promoted fluorapatite mineral formation in the subsurface lesion with both the control and the CPP-ACP gums [Reynolds, 1998]. The formation of fluorapatite would also help explain why the control and CPP-ACP-treated lesions were remineralized in situ with mineral that was relatively more stable to acid challenge than the original enamel.

The results of this study therefore show that sugar-free gum containing CPP-ACP is superior to an equivalent gum not containing CPP-ACP in remineralization of enamel subsurface lesions in situ with mineral that is more resistant to subsequent acid challenge.

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