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Remineralisation by Chewing Sugar-Free Gums in a Randomised, Controlled in situ Trial Including Dietary Intake and Gauze to Promote Plaque Formation

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Key Words
Caries in situ models · Casein phosphopeptide amorphous calcium phosphate · Enamel subsurface lesion remineralisation · Gauze · Plaque composition · Sugar-free chewing gum

Abstract
Remineralisation has been shown to be an effective mechanism of preventing the progression of enamel caries. The aim of this double-blind, randomised, cross-over in situ study was to compare enamel remineralisation by chewing sugar-free gum with or without casein phosphopeptide amorphous calcium phosphate (CPP-ACP) where the enamel lesions were exposed to dietary intake and some were covered with gauze to promote plaque formation. Participants wore removable palatal appliances containing 3 recessed enamel half-slabs with subsurface lesions covered with gauze and 3 without gauze. Mineral content was measured by transverse microradiography, and plaque composition was analysed by real-time polymerase chain reaction. For both the gauze-free and gauze-covered lesions, the greatest amount of remineralisation was produced by the CPP-ACP sugar-free gum, followed by the gum without CPP-ACP and then the no-gum control. Recessing the enamel in the appliance allowed plaque accumulation without the need for gauze. There was a trend of less remineralisation and greater variation in mineral content for the gauze-covered lesions. The cell numbers of total bacteria and streptococci were slightly higher in the plaque from the gauze-covered enamel for 2 of the 3 treatment legs; however, there was no significant difference in *Streptococcus mutans* cell numbers. In conclusion, chewing sugar-free gum containing CPP-ACP promoted greater levels of remineralisation than a sugar-free gum without CPP-ACP or a no-gum control using an in situ remineralisation model including dietary intake irrespective of whether gauze was used to promote plaque formation or not.

The chewing of sugar-free gum has been shown to promote the remineralisation of enamel subsurface lesions in situ [Leach et al., 1989] and to reduce the incidence of dental caries in clinical trials [Kandelman and Gagnon, 1990; Makinen et al., 1995; Beiswanger et al., 1998]. The anticariogenic effect of chewing sugar-free gum has been attributed to salivary flow stimulation [Jenkins and Ed-

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gar, 1989; Dawes and Dong, 1995], resulting in an increase in plaque pH [Yankell and Emling, 1989; Macpherson and Dawes, 1993], an increase in the availability of calcium and phosphate ions [Leach et al., 1989] and the clearance of food debris [Addy et al., 1982]. Casein phosphopeptide amorphous calcium phosphate (CPP-ACP), a stabilised source of bioavailable calcium and phosphate ions, has been added to sugar-free gum to enhance its anticariogenic properties [Shen et al., 2001]. The ability of CPP-ACP to enhance the remineralisation of enamel subsurface lesions has been shown in several in situ studies [Shen et al., 2001; Reynolds et al., 2003; Cai et al., 2007; Manton et al., 2008]. Additionally, a sugar-free gum containing CPP-ACP significantly slowed the progression and enhanced the regression of approximal caries relative to a control sugar-free gum in a 24-month randomised controlled clinical trial [Morgan et al., 2008].

Most of the in situ studies investigating the remineralisation effect of CPP-ACP in sugar-free gum have utilised a removable palatal appliance that contained demineralised subsurface lesions in slabs recessed in the appliance to encourage plaque accumulation. In this model, the appliance was removed during eating or drinking to avoid the confounding effect of participants' diet on remineralisation [Shen et al., 2001; Reynolds et al., 2003; Cai et al., 2007; Manton et al., 2008]. This in situ model has shown a fluoride dose response [Reynolds et al., 2008], and the remineralisation results for a CPP-ACP-containing sugar-free gum [Shen et al., 2001] were found to be predictive of the efficacy obtained for the gum in a randomised, controlled clinical trial [Morgan et al., 2008]. Alternative in situ remineralisation models have been used in which the enamel slabs are retained in the mouth during normal dietary intake and the slabs are covered with a layer of gauze to encourage plaque accumulation. Although there has not been a direct comparison of results obtained with each of these in situ models, it has been claimed that exposure to normal dietary intake and inclusion of a gauze covering are necessary components of any in situ model used to assess the remineralisation potential, in spite of the expected increased variance and decreased power of the study [Leach et al., 1989].

Therefore, the aim of this study was to compare the remineralisation effect of a CPP-ACP-containing sugar-free chewing gum with that of a regular sugar-free chewing gum and a no-gum control group in an in situ model where the appliance was exposed to the normal diet to provide a regular acid challenge and some of the enamel slabs were covered with gauze to promote plaque formation.

Subjects and Methods

Participants

Nine healthy adults living in a fluoridated community participated in this double-blind, randomised, crossover study. The participants were recruited from staff and students of the Melbourne Dental School, University of Melbourne. Study inclusion criteria were: age 18–60 years; at least 22 natural teeth; gum-stimulated salivary flow rate $\geq$1.0 ml/min and unstimulated whole salivary flow rate $\geq$0.2 ml/min. Subjects were additionally excluded if: their occlusion precluded fabrication of an in situ appliance; they were currently using antibiotics or medications that may affect salivary flow rate; they had current caries activity or periodontal disease, a history of rampant caries or any gross oral pathology. This study protocol was approved by the University of Melbourne Human Research Ethics Committee (No. 0932529). The number of participants required for this study was based on the results of a pilot study. The required sample size was calculated using the G*Power version 3.1 (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/) sample size package and was based on a repeated measures analysis of variance with 1 within-subject factor with 3 levels, an effect size of 0.97, a correlation, ρ, between any pair of treatment means of 0.5 and a non-sphericity correction, ε, of 0.5. The effect size of 0.97 was based on detecting differences between $\Delta Zd - \Delta Zr$ means of 50 (no-gum control), 225 (sugar-free gum without CPP-ACP) and 500 (sugar-free gum with CPP-ACP), and a common standard deviation of 200 within groups. These values were obtained from the pilot study. The non-sphericity correction adjusts for heterogeneity in the variances of the repeated measures. With a 5% significance level and a power of 90%, at least 6 subjects were required. To allow for subject non-compliance and drop-out, and since it is preferable to have a balanced design for the statistical analysis (equal numbers of subjects should be given each treatment in each time period) 9 subjects (3 per treatment sequence) were recruited to participate in the study.

Intra-Oral Appliances

A removable palatal acrylic appliance covering the first premolars to the most posterior teeth and retained by 4 narrow-gauge stainless-steel circumferential clasps was fabricated for each subject. Three windows on each side of the appliance accommodated 3 enamel half-slabs containing demineralised subsurface lesions (6 half-slabs per appliance). The enamel half-slabs were inset into larger windows in the undersurface of the appliance to expose the subsurface lesions in the smaller windows on the exposed surface of the appliance. Three of the 6 half-slabs on one side were covered by multifilament polyester surgical gauze (PETKM3002, SurgicalMesh™, Brookfield, Conn., USA). The side containing the 3 half-slabs covered by gauze was randomly chosen for each subject but remained the same for each treatment. The half-slabs with and without overlying gauze were recessed into the appliance by 1.5 mm to protect the dental plaque accumulating on the slab/gauze. The slabs and gauze were securely attached to the undersurface of the appliance with heavy body polyvinyl siloxane impression material (Kerr, North Ryde, N.S.W., Australia).

Preparation of Enamel Subsurface Lesions

Extracted third molars were obtained from oral surgeons and general practitioners in the Royal Dental Hospital of Melbourne

or private practice. The teeth were sterilised and stored in a 10% (v/v) neutral phosphate-buffered formalin solution for 2 weeks. Sound relatively planar buccal and lingual surfaces with minimal cracking, staining and fluorosis (as viewed under a dissecting microscope) were selected, then polished wet to a mirror finish using Soflex (3M) polishing discs on a slow-speed contra-angle dental handpiece. Each polished surface was then sawn from the tooth as an approximately 8 × 4 mm slab, using a water-cooled diamond blade saw, and the whole slab was covered with acid-resistant nail varnish except for 2 (occlusal and gingival) mesiodistal windows (approx. 1 × 7 mm) separated from each other by about 1 mm. Lesions were created in the enamel windows by mounting each slab onto the end of a 3- to 4-cm stick of yellow dental sticky wax and immersing in 40 ml of unagitated demineralisation buffer [White, 1987], consisting of 80 ml/l Noverite K-702 (polycacrylate, Lubrizol Corporation, Wickliffe, 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 4 days at 37°C with solutions changed after 2 days. This demineralisation procedure produced consistent subsurface lesions with intact surface layers, as evaluated by microradiography of sections of the artificial lesions. After demineralisation, each enamel slab was sawn through the midline of each window into two 4 × 4 mm slabs, and the cut surface of each slab was covered with nail varnish. One half of each slab was retained as the demineralisation control (control half-slab) and stored in a humidified environment. The other half of the enamel slab was inset into the intra-oral appliance (test half-slab) with polyvinyl siloxane impression material as described above. The enamel half-slabs and gauze were removed and replaced with new enamel half-slabs and sterile gauze at the beginning of every test period, thus bringing the total number of enamel half-slabs used to 162. After each treatment period, the test enamel half-slabs with and without overlying gauze were removed. All half-slabs from each side were used for plaque analysis using real-time polymerase chain reaction (RT-PCR) to determine total bacterial count, total streptococcal count, *Streptococcus sanguinis* and *Streptococcus mutans* count and afterwards were paired with their control half-slabs and embedded, sectioned and analysed to determine mineral content changes.

Study Products and Randomisation

Subjects were randomly assigned to one of the following groups: (1) sugar-free gum containing 18.8 mg/g CPP-ACP; (2) regular sugar-free gum without CPP-ACP, and (3) no-gum chewing control. The sugar-free chewing gums with and without CPP-ACP were prepared by the Clinical and Consumer Group of Kraft-Adams Worldwide Research and Development (N.J., USA). The gums were provided as coded products in sealed packages and stored at room temperature.

The treatment sequences were generated by the Statistics and Analytical Sciences Group of Kraft-Adams Worldwide Research and Development and assigned to subject codes. The study team then randomly assigned participants to a subject code. The gum allocation was only divulged by the study sponsor after all the data had been acquired.

Study Protocol

The study was conducted at the Royal Dental Hospital of Melbourne, Melbourne, Australia, over a 12-month period. Prior to the first treatment period, all subjects wore their appliance without the enamel slabs or gauze attached for 24 h a day for 3 consecutive days to ensure that their appliance fitted comfortably. Three days later, the study commenced. Participants were allocated to one of the three groups according to the random code and wore their appliance with enamel slabs and polyester gauze attached for 24 h a day (except during oral hygiene procedures) for 14 days. At the end of this 14-day period, they rested from the study for 1 week while new enamel slabs and new polyester gauze were attached to the appliance. Participants were then allocated their next randomly assigned treatment, and the process was repeated until each participant completed each of the 3 regimes. Participants chewed 1 slab of their allocated gum product for 20 min, 5 times a day on each of the 14 days after the following meals and snacks: breakfast; morning tea; lunch; afternoon tea; dinner. Participants did not eat or drink anything when chewing their allocated gum and kept a diary of times and duration of gum chewing and times when appliances were removed for oral hygiene procedures. Participants were instructed to maintain their usual diet and oral hygiene procedures for the duration of the study. The appliances were only removed briefly by the participants twice daily when they brushed their own teeth, and at one of these times they also cleaned their appliance. All participants used a standard fluoride toothpaste (0.245% w/v NaF, Crest Cavity Protection, Procter and Gamble, Cincinnati, Ohio, USA) to clean their own teeth for the duration of the study. Participants were instructed to clean only the palatal fitting surface (undersurface) of their appliance using a fluoride-free denture cleanser paste (Dentu-Creme, Polident, GlaxoSmithKline, Moon Township, Pa., USA) and toothbrush and not to brush the exposed side of the appliance. After cleaning the appliance, they gently rinsed both the exposed surface and the undersurface areas with a plastic squeeze bottle containing distilled de-ionised water. When the participants returned to the clinical site with their appliances at the conclusion of each 14-day treatment period, the research team removed the half-slabs for plaque and mineral content analysis.

Sectioning, Microradiography and Microdensitometric Image Analysis

Each pair of enamel half-slabs (remineralisation half-slab paired with its demineralisation control half-slab) had the nail varnish carefully removed and was embedded in transparent cold-curing methacrylate resin (Paladur, Heraeus Kulzer, Germany). Sections 200–300 μm thick were cut from the embedded half-slabs perpendicularly to the lesion surface through the midline of both half-lesions using an internal annulus saw microtome (Leitz 1600, Ernst Leitz, Wetzlar, Germany). The sections were then lapped down to 90 ± 5 μm using a RotoPol21/RotoForce4 lapping instrument (Struers, Denmark) with 1,200-grit lapping paper. Each section, which contained the remineralised half-lesion and the paired demineralised control half-lesion from the same enamel slab, was radiographed along with an aluminium step wedge of increments 7 × 37.5 μm thick using Microchrome High Resolution glass plates (HTA Enterprises, Microchrome Technology Products, San Jose, Calif., USA) and nickel-filtered copper Kα radiation as described previously [Shen et al., 2011].

Radiographic images of the lesions were viewed via transmitted light through a Leica DM 5500B microscope (Leica, Germany). The images were acquired by a Progres® MF scan digital camera (Jentopik, Jena, Germany) under the control of Image-Pro

Plus version 7.0 imaging software on a Sci-Tech Imaging Workstation (Sci-Tech, Preston, Vic., Australia). Images of the lesions and the neighbouring areas of sound enamel were scanned using the program's line luminance function that gave readings in grey values. An area free of artefacts or cracks was selected for analysis. Each scan comprised 200 readings taken from the tooth surface to sound enamel. The start and end of the lesion were defined as the points where the mineral density was 20 and 95% that of the sound enamel [ten Cate et al., 1996]. The 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 images lay within the linear portion of the step wedge curve, and linear regression was used to convert the grey value data into values of equivalent thicknesses of aluminium. The section thicknesses were 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 apatite mineral (131.5, 11.3 and 260.5, respectively). The image of the median strip between the two lesions was scanned 6 times and averaged to give a control sound-enamel densitometric profile. The lesion images (remineralisation windows and demineralisation 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 but avoiding any irregularities commonly found at the lesion edges, and the volume percent mineral content profiles were computed.

RT-PCR Analysis of Plaque

The enamel half-slabs (including gauze when used) were removed from the appliance and immediately placed into 500 μl of 20 mM Tris buffer and subjected to sonication in a water bath for 10 min to remove the plaque biofilm. The enamel half-slab was then removed, and the plaque sample frozen in liquid N_2 and stored at –80°C prior to analysis. DNA was extracted from plaque using the Power Biofilm DNA isolation kit (Geneworks, Thebarton, S.A., Australia) and a bead beater to ensure DNA extraction from Gram-positive bacteria. DNA concentration was determined using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Scoresby, Vic., Australia). DNA concentration in nanograms per microlitre was then converted to the equivalent number of cells per microlitre for use as RT-PCR standards using chromosome size (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000337240). DNA from plaque samples was diluted to 10 ng/μl prior to RT-PCR in order to limit inhibition of the PCR reaction due to high concentrations of DNA. Four sets of oligonucleotide primers were used targeting the glucosyltransferase B gene of *S. mutans*, the D-alanine:D-alanine ligase gene of *S. sanguinis*, and the 16S rRNA gene for all streptococci and total bacteria (suppl. table 1). RT-PCR conditions were optimised for the Corbett Rotor Gene P (Corbett Research, Mortlake, N.S.W., Australia). Reactions were carried out in triplicate, in a 25-μl reaction volume consisting of 12.5 μl of Platinum L SYBR Green qPCR Supermix UDG (Invitrogen, Mt Waverley, Vic., Australia), 9.5 μl DNase-free deionised water and 200 nM final concentration of forward and reverse primer and 2 μl template DNA. RT-PCR reaction conditions for primer pairs consisted of an initial heating step at 50°C for 2 min, initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. Fluorescence data were collected immediately following the extension step of each cycle. Specificity of the primer pairs was confirmed by melt curve analysis by heating from 72 to 95°C in 0.2°C increments. Melting peaks were compared to the bands obtained following agarose gel electrophoresis, where 10 μl of the PCR product was run on a 1.5% agarose gel with a 100-bp DNA ladder. The amplification products were visualised and photographed under a UV light transilluminator. Reactions for which melting peaks indicated the presence of non-specific amplification product were excluded from analysis. Numbers of bacterial cells were determined by reference to a standard curve created using a 10-fold serial dilution of a mix of DNA from equivalent cell numbers of *S. mutans* and *S. sanguinis* grown in batch culture. When determining the total number of streptococcal cells, it was assumed that the average number of 16S rRNA genes per streptococcus genome was 5. This was determined by averaging the 16S rRNA gene copy number for streptococci listed in the Ribosomal RNA Database (accessed October 28, 2010) [Lee et al., 2009]. When determining the total cell number of bacteria, it was assumed that the average number of 16S rRNA genes per genome (cell) in bacteria from dental plaque was also 5. This was determined by cross-referencing organisms listed in both the Human Oral Microbiome Database [Chen et al., 2010] (accessed October 28, 2010) and the Ribosomal RNA Database and then averaging the 16S rRNA gene copy numbers obtained. All data analysis was carried out using Corbett Rotor Gene application software, version 6.1, build 93 (Qiagen, Doncaster, Vic., Australia).

RT-PCR analysis was also conducted on supragingival plaque collected from all participants after the 3 treatment legs. All participants abstained from all oral hygiene procedures for 4 days. At the end of this period, plaque was collected from 4 sites on the buccal and lingual surfaces of the maxillary molars with a sterilised curette and analysed as described above.

Data Analysis and Statistical Considerations

The mineral content profile of each enamel slab's demineralised and remineralised lesion was compared with the median sound enamel mineral content profile of the same section. The difference between the areas under the densitometric profile of the demineralised lesion and the median sound enamel, calculated by trapezoidal integration, was represented by ΔZd. The difference between the areas under the densitometric profile of the remineralised lesion and the median sound enamel, calculated by trapezoidal integration, was represented by ΔZr. These ΔZ values were then used to calculate total mineral loss or gain (ΔZd – ΔZr) and percent mineral change (%R), i.e. $\%R = 100 \times (\Delta Zd - \Delta Zr)/\Delta Zd$. A negative ΔZd – ΔZr (or %R) value indicated mineral had been lost and therefore the lesion had progressed, whereas a positive value indicated mineral had been gained and therefore the lesion had regressed. Lesion depth (LD) was also measured for each lesion image and the change after treatment calculated as ΔLD = LDd – LDr. A negative ΔLD indicated an increase in LD. The effects of treatment and gauze on enamel remineralisation were compared between slabs with and without gauze using the integrated mineral gain/loss, ΔZd – ΔZr, as the primary outcome measure. Secondary outcome variables were %R and ΔLD.

The outcome measures from the RT-PCR analysis of the plaque samples were cell counts of total bacteria, all streptococci, *S. sanguinis* and *S. mutans*. The relative amounts of all streptococci, *S. sanguinis* and *S. mutans* as a percentage of total bacterial counts and *S. sanguinis* and *S. mutans* as a percentage of all strep-

Table 1. Enamel subsurface lesion parameters after exposure to sugar-free chewing gum containing CPP-ACP, sugar-free chewing gum without CPP-ACP or a no-gum control (means ± SD)

Treatment	Gauze				No gauze			
	ΔZd	ΔZd – ΔZr	ΔLD	%R	ΔZd	ΔZd – ΔZr	ΔLD	%R
No-gum control (n = 9)	3,854 ± 427	–136 ± 218[d]	–11.2 ± 10.6[e]	–3.6 ± 5.4[d]	3,769 ± 272	47 ± 107[d]	–1.5 ± 7.4[e]	1.3 ± 2.5[d]
Sugar-free gum without CPP-ACP (n = 9)	3,913 ± 345	152 ± 201[b]	–1.9 ± 6.5[a]	3.7 ± 5.3[b]	3,942 ± 257	270 ± 65[a]	0.1 ± 3.7	6.5 ± 2.2[a]
Sugar-free gum with CPP-ACP (n = 8)	4,230 ± 373	595 ± 207[b, c]	5.3 ± 3.7[b]	13.5 ± 4.8[b, c]	4,145 ± 595	709 ± 239[b, c]	8.1 ± 4.3[a]	14.6 ± 6.0[b, c]

ΔLD = LDd – LDr; the mean LDd (initial lesion depth) was 125 ± 7 μm. [a] $p < 0.05$, [b] $p < 0.005$: significantly different from no-gum control after Sidak adjustment for multiple comparisons; [c] $p < 0.005$: significantly different from sugar-free gum without CPP-ACP after Sidak adjustment for multiple comparisons; [d] $p < 0.05$, [e] $p < 0.001$: significantly different between gauze and no gauze for no-gum control.

tococcal counts were also calculated for each plaque sample. $\log_{10}$ transformations of the bacterial count data and complementary log-log transformations of the relative abundance data were used prior to analysis.

The unit of analysis was the participant. The outcome measures were determined for the demineralised and remineralised lesions from each enamel slab, and the resultant values averaged by participant, treatment and presence of gauze. Descriptive statistics (mean and standard deviation) were calculated for all outcome measures, and the data were analysed using a linear mixed modelling approach [Verbeke and Molenberghs, 2000]. Fixed effects included in the models were treatment, gauze, treatment by gauze interaction and treatment period; participant was included as a random effect. Gauze was also specified as a repeated measures effect with compound symmetry covariance structure to account for the correlated data as the appliances included enamel slabs both with and without gauze at each treatment period. Post hoc comparisons of treatment differences were performed on the marginal means using the Sidak adjustment for multiple comparisons. Modelling assumptions were checked using residual and normal probability plots. p values less than 0.05 were regarded as being statistically significant. All analyses were conducted using either SPSS (version 18.0; SPSS Inc., Chicago, Ill., USA) or Stata (version 10.1; Stata Corp. LP, College Station, Tex., USA) statistical software.

Results

Participants

Thirteen eligible individuals were screened of which 4 participants were excluded because their occlusion precluded a comfortably fitting palatal appliance. The mean participant age was 38 years (range 21–52 years) with 4 females and 5 males. The mean unstimulated and stimulated whole salivary flow rates of the participants were 0.57 ± 0.31 and 3.05 ± 1.48 ml/min, respectively. All participants completed the study. The data for 1 participant in 1 treatment leg was not included in the analysis as the treated enamel half-slabs were damaged (online suppl. fig. 1).

Remineralisation Data

The mean values for the enamel subsurface lesion parameters and their relative change after exposure to the sugar-free gum with and without CPP-ACP and the no-gum control are shown in table 1. The demineralised control lesions all had a similar initial integrated mineral loss (ΔZd) prior to exposure to the oral environment. For both the gauze-free and gauze-covered lesions, the greatest amount of remineralisation was provided by chewing the sugar-free gum containing CPP-ACP, followed by the sugar-free gum without CPP-ACP and then the no-gum chewing control. Gauze-covered lesions in the no-gum chewing group were found to undergo further demineralisation during the treatment period as shown by a significant decrease in total mineral content (ΔZd – ΔZr) and increase in lesion depth (table 1). The gauze-covered lesions all tended to undergo less remineralisation than the gauze-free lesions though this difference was only statistically significant for the no-gum control group (table 1).

Microbiological Data

Plaque was seen to develop in the appliance recesses on both the gauze-free and gauze-covered enamel half-slabs, which was confirmed by RT-PCR analysis (table 2). The RT-PCR analysis of the plaque samples overlying the enamel half-slabs found no differences in numbers of total bacteria, total streptococci, *S. sanguinis* and *S. mutans* between the various treatments (table 2). However, the presence of gauze tended to increase the total cell number

Table 2. Bacterial composition of supragingival plaque and plaque overlying the enamel half-slabs in the appliance exposed to sugar-free chewing gum containing CPP-ACP, sugar-free chewing gum without CPP-ACP or a no-gum control (means ± SD)

Bacterial parameter	No gum (n = 9)		Sugar-free gum without CPP-ACP (n = 9)		Sugar-free gum with CPP-ACP (n = 8)		Supragingival plaque
	no gauze	gauze	no gauze	gauze	no gauze	gauze	
$\log_{10}$ (number of total bacterial cells)	7.7 ± 0.5[a]	8.3 ± 0.3[a]	7.9 ± 0.4	8.0 ± 0.4	7.8 ± 0.5[c]	8.2 ± 0.7[c]	8.4 ± 0.4
$\log_{10}$ (number of streptococcal cells)	7.2 ± 0.5[a]	7.7 ± 0.3[a]	7.4 ± 0.4	7.5 ± 0.5	7.4 ± 0.5[c]	7.9 ± 0.5[c]	7.8 ± 0.5
Percent streptococci/total bacteria	43.1 ± 25.6	32.9 ± 16.3	31.4 ± 11.5	33.0 ± 18.8	50.9 ± 22.8	57.2 ± 22.6	22.6 ± 19.5
$\log_{10}$ (number of *S. sanguinis* cells)	5.0 ± 1.1	5.1 ± 1.0	5.3 ± 0.9	5.0 ± 1.3	5.0 ± 1.3[c]	5.2 ± 1.4[c]	6.8 ± 0.8
Percent *S. sanguinis*/total bacteria	0.6 ± 0.7[a]	0.5 ± 1.1[a]	1.1 ± 1.8[b]	2.4 ± 6.2[b]	4.4 ± 7.5[c]	1.4 ± 2.8[c]	8.7 ± 17.0
Percent *S. sanguinis*/streptococci	1.5 ± 1.7	1.3 ± 2.2	3.6 ± 6.4	4.6 ± 10.5	7.3 ± 9.6[c]	2.9 ± 6.2[c]	19.5 ± 24.7
$\log_{10}$ (number of *S. mutans* cells)	4.7 ± 2.1	5.4 ± 1.8	5.5 ± 1.8	5.5 ± 1.9	5.2 ± 1.7	5.4 ± 2.5	4.0 ± 2.6
Percent *S. mutans*/total bacteria	4.4 ± 11.3	2.9 ± 6.9	6.0 ± 10.1	4.4 ± 6.8	2.9 ± 4.6	2.0 ± 2.1	0.2 ± 0.2
Percent *S. mutans*/streptococci	5.9 ± 12.1	10.4 ± 18.9	21.0 ± 30.7	21.8 ± 36.7	6.9 ± 9.2	7.3 ± 10.8	1.1 ± 1.9

[a, b, c] $p < 0.05$: similarly marked values in the same row are significantly different.

of bacteria and streptococci with this reaching significance in the no-gum and CPP-ACP sugar-free gum groups (table 2). There were no significant differences in the cell numbers of *S. mutans* and the percentage of *S. mutans* of total bacteria and total streptococci in the plaque with or without gauze.

The 4-day-old supragingival plaque obtained from the participants had a lower percentage of streptococci compared with the 14-day-old plaque from the appliance. Additionally, the percentage of *S. sanguinis* per total bacteria or total streptococci in the supragingival plaque was significantly higher than that in the plaque from the appliance. The percentage of *S. mutans* per total bacteria or total streptococci in the supragingival plaque was significantly lower than that in the plaque from the appliance (table 2).

Adverse Events

A number of non-serious adverse events (treatment-related or unrelated) were recorded for 5 of the 9 participants. The majority were related to the appliance and included minor discomfort or ulceration. No serious adverse events were recorded, and no participants withdrew due to adverse events.

Discussion

Intra-oral remineralisation models are important for the development and testing of novel anticariogenic agents prior to conducting expensive and time-consuming clinical trials. This study tested the remineralisation efficacy of a CPP-ACP-containing sugar-free gum compared with a sugar-free gum without CPP-ACP and a no-gum control in a palatal appliance in situ model including normal dietary intake and gauze to promote plaque formation. A gauze covering over enamel subsurface lesions in an in situ model has been recommended as a method of encouraging plaque formation [Leach et al., 1989]; therefore, in the current study we included 3 enamel half-slabs with a gauze covering and 3 without to determine whether there was a promotion of plaque formation by the gauze. All enamel half-slabs with or without gauze were recessed in the appliance to allow the development of a uniform biofilm [Walker et al., 2009].

For both the gauze-free and gauze-covered lesions, the greatest amount of remineralisation was provided by chewing the sugar-free gum containing CPP-ACP, followed by the sugar-free gum without CPP-ACP and then the no-gum chewing control (table 1). This is consistent with previous studies that have shown that a sugar-free gum containing CPP-ACP is superior to a sugar-free gum without CPP-ACP in the remineralisation of enamel subsurface lesions [Shen et al., 2001; Morgan et al., 2008]. This has been attributed to the localisation of bio-available calcium and phosphate ions at the tooth surface that can depress enamel demineralisation and promote enamel remineralisation [Cochrane et al., 2010].

The palatal appliance used in this study was similar in design to a palatal appliance used in previous in situ studies [Shen et al., 2001; Reynolds et al., 2003; Cai et al., 2007; Manton et al., 2008; Walker et al., 2009] but different in

that the enamel half-slabs were fixed in place from the undersurface of the appliance using polyvinyl siloxane. Furthermore 3 of the 6 enamel half-slabs in the appliance were covered with polyester gauze, and the appliance was worn at all times including meals and removed only during oral hygiene procedures twice per day. In one of the previous in situ studies [Shen et al., 2001], a sugar-free gum containing 18.8 mg CPP-ACP was shown to promote a significantly greater remineralisation of subsurface lesions (18.2 ± 1.7% remineralisation) compared with a normal sugar-free gum (9.0 ± 1.4% remineralisation) and no-gum chewing (3.3 ± 1.0%) when chewed 4 times per day for 20 min. The levels of remineralisation obtained in the study of Shen et al. [2001] were slightly higher and the variances lower than that of the current study. The major difference between these two studies was that the appliances were removed at meal times in the study of Shen et al. [2001]. In the current study, participants maintained their usual diet, and therefore lesions were subjected to the different cycles of de- and remineralisation based on the form and frequency of the participants' dietary choices. This highlights the increased variance introduced into a study by exposing the enamel half-slabs to the participants' diet. An in vitro acid challenge after a treatment phase can be incorporated to test the acid resistance of deposited mineral in a controlled fashion [Iijima et al., 2004; Cai et al., 2007]. Notwithstanding the influence of the diet, significant differences were still found between no-gum chewing, the sugar-free gum without CPP-ACP and the CPP-ACP-containing sugar-free gum in agreement with the results of previous in situ models not including normal dietary intakes [Shen et al., 2001; Cai et al., 2007].

The gauze-covered lesions all tended to undergo less remineralisation than the gauze-free lesions; however, the difference was only statistically significant for the no-gum control group (table 1). Zero [1995] found that gauze-free lesions consistently had a greater remineralisation response than gauze-covered lesions. In the model of Zero [1995], the bovine enamel was mounted flush with the buccal flange so that no recess was present to protect and allow the development of plaque. The appliance in the current study utilised a 1.5-mm-deep recess to allow plaque formation on the enamel surface protected from the abrasive forces of food and the tongue. A difference in remineralisation between gauze-covered and gauze-free lesions can either relate to increased demineralisation, decreased remineralisation or a combination of both. The RT-PCR analysis of the plaque indicated that gauze-covered lesions did exhibit slightly higher total bacterial cell numbers for 2 of the 3 treatments but no differences in levels of *S. mutans* were detected between gauze and no-gauze plaques. It is possible that the presence of an unnatural covering over the enamel lesions acts to impede diffusion of saliva hence lowering its ability to prevent demineralisation and promote remineralisation [Dawes et al., 1989; Mellberg, 1992]. This may explain why the gauze-covered lesions in the no-gum chewing group underwent further demineralisation during the treatment period with an increase in lesion depth.

The gauze covering of enamel subsurface lesions has been proposed as a method of encouraging plaque formation [Leach et al., 1989] and controlling plaque thickness [Zero, 1995]. The primary contention is that gauze-free lesions may be plaque free, thereby resembling smooth surfaces and not resembling an at-risk site [Zero, 1995]. Other authors have recessed the enamel slabs below the acrylic flange as in the current study to protect the plaque [Corpron et al., 1986; Dijkman et al., 1986; Cai et al., 2007; Walker et al., 2009]. The current study clearly shows that a recess can be used to protect plaque as RT-PCR analysis confirmed that plaque was present and it could be visualised on the enamel half-slabs. The non-gauze plaque also developed the same level of *S. mutans* as the gauze-related plaque. The gauze may offer the plaque protection from the abrasive forces in the mouth if there is no other protection for the plaque but it has been postulated that this may lead to the formation of a less natural plaque [Zero, 1995]. Hence, allowing the development of a thickness-controlled biofilm by the use of a recess in the appliance may be preferable to the use of an unnatural covering to promote plaque formation.

To test whether the composition of the plaque on the appliance was similar to natural plaque, 4-day-old supragingival plaque was obtained from the participants. It was found that the supragingival plaque had a higher percentage of *S. sanguinis* and a lower percentage of *S. mutans* compared with the 14-day-old plaque from the appliance (table 2). Due to ethical reasons supragingival plaque was collected after 4 days rather than 14 days which would have corresponded to the age of the plaque on the appliance enamel. Therefore, differences in the composition of the supragingival plaque compared with the appliance enamel plaque may be related to biofilm maturation changes with time. CPP-ACP has been suggested to alter biofilm composition [Neeser et al., 1994; Rose, 2000; Rahiotis et al., 2008] but in the current study no significant differences in total bacterial cell numbers or composition were found between treatments. Howev-

er, this may relate to the protection of the plaque by the recess/gauze in the appliance design and the low level of CPP-ACP in the sugar-free gum.

Ultimately, an in situ model is used to serve as a bridge between in vitro experiments and clinical trials [Zero, 1995]. Therefore, an in situ model should be predictive of clinical trial results. Many clinical studies have now been conducted demonstrating the benefits of sugar-free gum compared with no-gum chewing [Kandelman and Gagnon, 1990; Makinen et al., 1995; Beiswanger et al., 1998]. Additionally, the benefits of a CPP-ACP sugar-free gum compared with a normal sugar-free gum has recently been shown [Morgan et al., 2008]. The results of the current in situ study for gauze-covered and gauze-free enamel lesions are in agreement with these clinical trial results as the CPP-ACP-containing sugar-free gum was found to be superior to the sugar-free gum without CPP-ACP which was in turn superior to no-gum chewing. Given that plaque was present on the recessed gauze-free enamel pieces and that the remineralisation values tended to be higher and the variance lower for the gauze-free data, then the gauze-free model should allow fewer participants to be used to differentiate remineralisation treatment effects. Furthermore as the gauze-free model does not involve an unnatural covering over the enamel lesion surface being studied, then the gauze-free model may be the preferred in situ model for screening novel remineralisation agents.

Conclusions

Sugar-free chewing gum containing CPP-ACP was found to promote higher levels of remineralisation than a sugar-free gum without CPP-ACP or a no-gum control using an intra-oral remineralisation model including dietary intake irrespective of whether gauze was used to promote plaque formation.

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