SCIENTIFIC ARTICLE

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Remineralization of enamel subsurface lesions *in situ* by sugar-free lozenges containing casein phosphopeptide-amorphous calcium phosphate

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Abstract

Background: The anticariogenic potential of casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) has been demonstrated using laboratory, animal and human *in situ* caries models. The aim of this study was to determine the effect of CPP-ACP incorporation into a sugar-free lozenge (pressed mint tablet) on enamel remineralization in a human *in situ* model.

Methods: The study utilized a double-blind, randomized, cross-over design with four treatments: (i) a lozenge containing 56.4mg (3 per cent w/w) CPP-ACP; (ii) a lozenge containing 18.8mg (1 per cent w/w) CPP-ACP; (iii) a lozenge not containing CPP-ACP; and (iv) a no lozenge nil-treatment control. Ten subjects wore removable palatal appliances with four, human-enamel, half-slab insets containing subsurface lesions. Lozenges were consumed, without chewing, four times per day for 14 days duration. After each treatment period the enamel slabs were removed, paired with their respective demineralized control, embedded, sectioned and subjected to microradiography and computer-assisted densitometric image analysis to determine the level of remineralization.

Results: The incorporation of CPP-ACP into the lozenge significantly increased enamel subsurface lesion remineralization with 18.8 and 56.4mg of CPP-ACP increasing remineralization by 78 and 176 per cent respectively, relative to the control sugar-free lozenge.

Conclusion: This study demonstrates that lozenges are a suitable vehicle for the delivery of CPP-ACP to promote enamel remineralization.

Key words: Enamel remineralization, sugar-free lozenge, casein phosphopeptide-amorphous calcium phosphate.

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INTRODUCTION

Phosphopeptides containing the phosphoseryl cluster sequence -Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu from the milk protein casein stabilize amorphous calcium phosphate as nanocomplexes in metastable solution.[1,2] These casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) have been demonstrated to have anticariogenic potential in laboratory, animal and human *in situ* experiments.[1-6] Recently, in a human *in situ* enamel remineralization study, Shen *et al.*[7] demonstrated that CPP-ACP delivered from a sugar-free chewing gum remineralized enamel subsurface lesions. Inclusion of CPP-ACP in the sugar-free gum promoted a dose-response increase in enamel subsurface lesion remineralization with 18.8 and 56.4mg increasing remineralization by 101 and 151 per cent respectively, relative to the control sugar-free gum.

Sugar-free lozenges (pressed mint tablets) stimulate saliva through gustatory stimulation[8,9] and completely dissolve when consumed such that they may also be an effective delivery vehicle for a safe and efficacious remineralizing agent. The aim of this study was to determine whether addition of CPP-ACP to a sugar-free lozenge would significantly increase enamel subsurface lesion remineralization in an *in situ* model.

MATERIALS AND METHODS

Subject recruitment

Ten (six males/four females) healthy adult subjects (mean age 34±6.6 years) from the staff and post-graduate students of the School of Dental Science, the University of Melbourne were recruited for this randomized, double-blind, cross-over study. Approval for the study was obtained from the University of Melbourne Human Research Ethics Committee and all subjects provided informed consent. An intra-oral examination confirmed that each subject 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. Unstimulated salivary flow rate was measured by

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instructing a subject to lean forwards with the head tilted downwards to allow saliva to flow into a pre-weighed centrifuge tube for two minutes. Lozenge stimulated whole salivary flow rate was measured by instructing a subject to consume a test lozenge (pressed peppermint tablet, the Warner Lambert Company, New Jersey, USA) for exactly two minutes without chewing. All the saliva produced was allowed to flow into a pre-weighed centrifuge tube. The volume of saliva collected in the two minute period was determined from the final weight of the centrifuge tube plus saliva.

Intra-oral appliances

Removable mid-palatal acrylic appliances covering the first premolars to the last tooth in the arch were prepared for each subject as described previously.[7]

Preparation of enamel subsurface lesions

Extracted human third molars were obtained from the Royal Dental Hospital of Melbourne. Any attached soft tissues were removed and the teeth were stored in an 18 per cent v/v formalin acetate solution for two weeks, washed three times with distilled water and then stored in 70 per cent (v/v) ethanol. Sound relatively planar buccal and lingual surfaces (as viewed under a dissecting microscope) were selected and the outer enamel surface was 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 with an enamel surface of 8x4mm^2, 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 (1x7mm^2) on the enamel surface separated from each other by 1mm.

Subsurface lesions were prepared in each window using the Carbopol method of White[10] as modified by Reynolds.[4] After demineralization each enamel slab was sawn through the midline of each window into two half-slabs with 4x4mm^2 enamel surfaces. One half-slab of each pair was retained as the demineralization control and stored in a humidified environment. The other enamel half-slab of the pair was inset into an intra-oral appliance as described by Shen *et al*.[7] Four enamel half-slabs were inset into each appliance, two on each side in bilateral troughs.[7]

Study protocol

The sugar-free lozenges were prepared by the Warner Lambert Company (New Jersey, USA) using CPP-ACP (Recaldent™) obtained from Bonlac Foods Limited (Melbourne, Australia). The lozenges were provided as coded products in sealed packages and were stored at room temperature. The code was held by an independent group and not released until all the data had been acquired. Four randomized treatments consisted of sugar-free lozenges (weight 1.75g) containing: (i) 18.8mg CPP-ACP; (ii) 56.4mg CPP-ACP; (iii) a lozenge not containing CPP-ACP; and (iv) a no lozenge nil-treatment control. The average time for the lozenge to dissolve in the mouth was eight minutes. The subjects were instructed not to chew the lozenge. For the nil-treatment control, the appliances were worn for the same total time period.

Subjects consumed the lozenges at the following times: 10am, 11:30am, 2pm and 3:30pm each day for 14 days. Subjects kept diaries of lozenge use and were instructed not to eat (except for the test lozenges), drink or perform oral hygiene procedures while wearing the appliances. When the appliances were not in the mouth they were stored in sealed moist plastic bags at room temperature. Subjects were instructed to rinse and clean their appliances using a fluoride-free denture cleanser paste and toothbrush provided. They were informed not to brush the area containing the enamel half-slabs and to maintain their normal diet and oral hygiene procedures. All subjects used standard fluoride dentifrice for the duration of the study.

After completion of each treatment period the enamel half-slabs were removed from the appliances for processing. The subjects used all four treatments in a cross-over design with at least one week wash out period between treatments. The treatments were randomized by subject and were double-blinded.

Enamel 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 and subjected to microradiography and computer-assisted densitometric analysis as described by Shen *et al*.[7] Images of the lesions and the neighbouring areas of sound enamel were scanned six times using the line luminance function of Optimate Version 6.2 that gives readings in Grey values between 0 and 256. Each scan comprised 200 readings taken from the tooth surface through the lesion to the 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 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 thickness of aluminium.

The section thickness was measured and the vol per cent mineral data computed using the equation of Angmar *et al*.[11] and the linear absorption co-efficients of aluminium, organic matter plus water and apatitic mineral (131.5, 11.3 and 260.5 respectively). The image of the median strip between the two lesions was scanned six times and averaged to give a control densitometric profile of sound enamel. The lesion images (remineralization window and demineralization control window) on the gingival and occlusal side of the median strip were similarly scanned, as close as possible to the median strip but avoiding any irregularities commonly found at the lesion edges, and

the vol per cent mineral profiles were computed. Macro programmes were written to control the imaging software and to transfer scanned data directly into Microsoft Excel.

Data analysis

The vol per cent mineral profile of each enamel half-slab's demineralized and remineralized lesion was compared with the median sound enamel vol per cent mineral profile 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 Zd. 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 Zr. These parameters were then converted to per cent change values after remineralization. As such, per cent remineralization (% R) represents the per cent change in Z values:

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

Data were statistically analysed using a one-way analysis of variance (ANOVA) with the Scheffe, a *posteriori* multiple-comparison test using software SPSS version 9.0.[12]

RESULTS

The average time for the lozenge to dissolve in the mouth was approximately eight minutes. The mean unstimulated salivary flow rate for the 10 subjects was 0.67±0.55ml/min. The mean salivary flow rate upon consuming the lozenge without chewing for two minutes was 2.48±0.98ml/min, which represented a 3-4 fold increase over the unstimulated flow rate.

The enamel subsurface lesions prepared for the *in situ* remineralization study were uniform with a mean lesion depth of 105.6±5.1µm and a mean Zd value of 3,070±441 vol% min.µm. The per centage enamel subsurface lesion remineralization (%R) effected by the sugar-free lozenges in the *in situ* study is presented in Table 1. The results represent the mean % R values for the 10 subjects and the data for each subject was obtained from 12 scans (six each for the occlusal and gingival lesions) performed on each section from the four half-slabs in each appliance.

Table 1. Effect of CPP-ACP in a sugar-free lozenge on remineralization of enamel subsurface lesions

Treatment/CPP-ACP dose (mg)	Sugar-free lozenge % R	% R
Nil-treatment	1.97±0.33[a, b]	–
0	7.03±0.65[b]	–
18.8	12.50±1.48[b]	5.47 (78%)[c]
56.4	19.39±1.69[b]	12.36 (176%)

a. Mean±SD (n=10).
b. Significantly different ($p<0.01$) from all other values in column similarly marked as shown using a Scheffe multiple comparison.
c. Percentage increase in remineralization relative to the control lozenge not containing CPP-ACP.

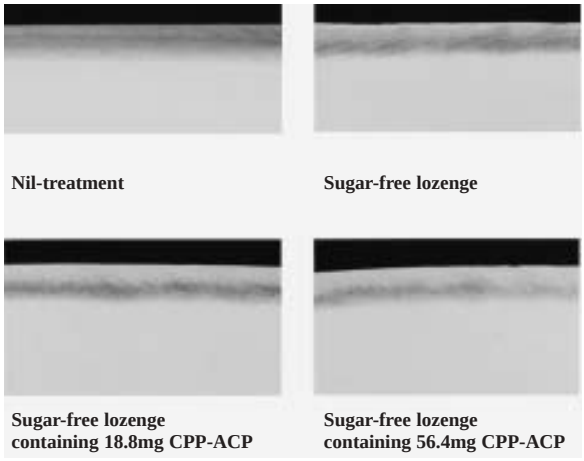

Fig 1. Representative microradiographs demonstrating enamel subsurface lesion remineralization by sugar-free lozenges containing CPP-ACP.

The incorporation of CPP-ACP into the lozenges resulted in a dose-response increase in enamel remineralization with 18.8 and 56.4mg of CPP-ACP significantly increasing remineralization by 78 and 176 per cent respectively, relative to the control sugar-free lozenge (Table 1). Representative microradiographs of enamel subsurface lesions after each treatment are shown in Fig 1. No significant correlation was found between individual unstimulated or stimulated salivary flow rates and enamel %R values obtained for any of the treatments.

DISCUSSION

In this *in situ* study we have shown that incorporation of CPP-ACP into sugar-free lozenges produced a dose-dependent increase in enamel subsurface lesion remineralization. The study further showed that consumption of the lozenges significantly increased salivary flow rate. The 3-4 fold increase in salivary flow rate upon consuming the lozenges for two minutes can be attributed to gustatory stimulation as the lozenges were not chewed. The stimulated salivary flow rate after two minutes of lozenge consumption was 2.48±0.98ml/min. This is similar to the value (2.92ml/min) obtained by Dawes and MacPherson[9] after two minutes of consumption of a lozenge (pressed mint tablet) like that used in the current study.

In the Dawes and Macpherson[9] study a comparison of saliva stimulation was made between lozenges and sugar-free chewing gum. Consumption of sugar-free chewing gum, through gustatory and mechanical stimulation, produced a significantly greater increase in salivary flow rate (3-4ml/min) over the first two minutes of consumption. This is similar to the salivary flow rate stimulated by chewing sugar-free gum for two minutes reported recently by Shen *et al.*[7] These studies therefore indicate that a lozenge is slightly less effective

than sugar-free chewing gum at stimulating saliva over the first two minutes of consumption. However, Dawes and Macpherson[9] showed that after two minutes of consumption, saliva stimulation by a lozenge was approximately equal to that of gum chewing. When the lozenge completely dissolved the salivary flow rate rapidly decreased towards the unstimulated level. In the current study the average time for the lozenge to completely dissolve was eight minutes. Hence, after this time salivary flow rate would be expected to decrease to the unstimulated level.

We have previously shown[7] that incorporation of CPP-ACP into sugar-free chewing gum significantly increased enamel remineralization in an *in situ* model. The results of the current study demonstrated that incorporation of CPP-ACP into a sugar-free lozenge also significantly increased enamel subsurface lesion remineralization in an *in situ* model identical to that used by Shen *et al*.[7] In fact, the increase in remineralization by the CPP-ACP was similar in both studies indicating that a sugar-free lozenge may be a suitable alternative vehicle to sugar-free gum for delivery of CPP-ACP.

CONCLUSION

In conclusion, we have demonstrated that incorporation of CPP-ACP into a sugar-free lozenge significantly increased remineralization of enamel subsurface lesions *in situ*, with 18.8 and 56.4mg of CPP-ACP increasing remineralization by 78 and 176 per cent respectively, relative to the control sugar-free lozenge.

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