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Increased remineralization of tooth enamel by milk containing added casein phosphopeptide-amorphous calcium phosphate

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Casein phosphopeptide amorphous calcium phosphate nanocomplexes (CPP-ACP) in chewing gum, lozenges and mouthrinses have been shown to remineralize enamel subsurface lesions in human *in situ* experiments. The aim of this double-blind, randomized clinical study was to investigate the capacity of CPP-ACP added to bovine milk to remineralize enamel subsurface lesions *in situ*. Ten subjects drank milk containing either 2·0 or 5·0 g CPP-ACP/l or a control milk whilst wearing removable appliances with enamel slabs containing subsurface demineralized lesions. Each 200 ml milk sample was consumed once a day for each weekday over three consecutive weeks. After each treatment and one weeks rest the subjects crossed over to the other treatments. At the completion of the treatments the enamel slabs were removed and remineralization determined using microradiography and microdensitometry. The results demonstrated that all three milk samples remineralized enamel subsurface lesions. However, the milk samples containing CPP-ACP produced significantly greater remineralization than the control milk. The remineralising effect of CPP-ACP in milk was dose-dependent with 2·0 and 5·0 g CPP-ACP/l producing an increase in mineral content of 70 and 148%, respectively, relative to the control milk. The differences in remineralization following exposure to the three milk samples were all statistically significant ($P<0·001$). In conclusion, this study shows that the addition of 2·0–5·0 g CPP-ACP/l to milk substantially increases its ability to remineralize enamel subsurface lesions.

Keywords: Casein phosphopeptide, amorphous calcium phosphate, milk, remineralization, tooth enamel.

Casein phosphopeptides (CPP) containing the sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu stabilize nanoclusters of amorphous calcium phosphate (ACP) in metastable solution (Reynolds, 1998; Reynolds et al. 1999). The multiple Ser(P) residues bind to forming nanoclusters of ACP in supersaturated solutions preventing growth of these clusters to the critical size for phase transformations. The anticariogenic potential of casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) in laboratory, animal and human *in situ* models has been well documented (Reynolds et al. 1995; Reynolds, 1997a, 1999; Rose, 2000; Shen et al. 2001). The remineralization of enamel subsurface lesions by CPP-ACP added to chewing gum (Shen et al. 2001), mouthrinses (Reynolds et al. 2003), and lozenges (Cai et al. 2003) has been demonstrated in human *in situ* models. Further, CPP-ACP has also been shown to remineralize enamel with mineral that is more resistant to acid challenge than normal tooth enamel mineral (Iijima et al. 2004). The proposed mechanism of action of CPP-ACP is related to its localization at the tooth surface where it buffers free calcium and phosphate ion activities maintaining a state of supersaturation with respect to tooth enamel, thereby preventing demineralization and facilitating remineralization (Reynolds, 1999).

Bovine milk has been shown to be anticariogenic in animal caries models and to remineralize enamel subsurface lesions *in vitro*, reviewed in Reynolds (1998). The anticariogenic properties of milk have been attributed to the casein, calcium and phosphate contents (McDougall, 1977; Mor & Rodda, 1983; Reynolds et al. 2003). However, in liquid milk the majority of the casein, and calcium and phosphate ions are bound in micelles and therefore upon consumption would not necessarily be available to promote remineralization of subsurface

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lesions in tooth enamel *in situ*. Addition of CPP-ACP to milk may substantially increase the bioavailable calcium and phosphate ion content and therefore may substantially increase the capacity of milk to remineralize enamel subsurface lesions *in situ*.

The aim of this study therefore was to investigate the capacity of CPP-ACP added to milk to remineralize enamel subsurface lesions using an established *in situ* model.

Materials and Methods

Subject recruitment

Ten healthy adult subjects with at least 22 natural teeth and normal salivary function were recruited from staff and students of the School of Dental Science, University of Melbourne. Ethics approval for this study was obtained from the University of Melbourne Human Research Ethics Committee. All subjects provided informed consent and were given an intra-oral examination. Stimulated salivary flow was measured during sugar-free gum chewing (Cadbury-Adams, Morris Plains, NJ) for exactly 2 min and the salivary flow rate measured. The stimulated salivary flow rate was in excess of 1·0 ml/min. Unstimulated salivary flow was in excess of 0·2 ml/min.

Intra-oral appliances

For each subject, a custom-made removable acrylic upper mid-palatal appliance retained by four stainless steel circumferential clasps, was fabricated. The appliance had two bilateral troughs (15 mm long, 7 mm wide and 2 mm deep), approximately 3 mm medial to the lateral margins of the appliance designed to house two enamel slabs each. The slabs were retained with sticky wax.

Preparation of enamel lesions

Extracted sound human third molars were collected and sterilized in formalin. Relatively planar buccal and/or lingual surfaces (8×4 mm^2) were then polished to a mirror finish with Soflex discs. The surfaces were then divided into occlusal and gingival mesiodistal windows (1×7 mm^2) separated by 1 mm (Reynolds, 1987) and the windows then cut from the rest of the crown. Subsurface lesions were created in the enamel windows by immersing the blocks in demineralising buffer containing 20 g Carbopol/ml, 500 mg hydroxyapatite/l and 100 mM-lactic acid (pH 4·8) as described by White (1987) for 4 d at 37 °C with a change of solution after 2 d. The windows were then sectioned through the midline to create two demineralized half slabs (4×4 mm^2) and the cut surfaces of each slab were covered with nail varnish. For each pair of half-slabs, one half-slab was stored in a humidified container (untreated slab) and the other half-slab was inset into the appliance (treated slab). Four half-slabs were inset into each appliance.

Preparation of CPP-ACP and milk samples

Casein phosphopeptide amorphous calcium phosphate (Recaldent™) was prepared by the previously described procedure (Reynolds, 1997b) and was provided by Recaldent Pty Ltd (Victoria, Australia). The Recaldent™ was supplied as a powder which contained 470 g CPP/kg, 143 g calcium/kg and 223 g phosphate/kg (Recaldent™ batch #841117). The major CPP in the preparation are the tryptic casein phosphopeptides including the sequence-Ser(P)-Ser(P)-Ser(P)-Glu-Glu- (Reynolds, 1999). The milk samples were prepared by Meiji Dairies Corporation (Japan). The milk was a normal bovine milk and the CPP-ACP was added prior to pasteurisation.

Study protocol

The study was conducted double blind. Three coded milk samples containing 2·0 g CPP-ACP/l, 5·0 g CPP-ACP/l, or no added CPP-ACP (control) were tested. The milk samples were stored in sealed bags at 4 °C for no longer than 60 d and were randomly allocated to the subjects. Once a day for 15 d, subjects with their appliances inserted, consumed 200 ml of milk by taking 10–15 sips over approximately 60 s, and then continued wearing the appliance for 40 min. During this period, the subjects refrained from drinking or eating anything other than the milk sample. After removal, each appliance but not the inset enamel blocks, was cleaned with a fluoride-free toothpaste and then rinsed with distilled deionized water. The appliances with the inset enamel blocks were then stored in a humid container at 37 °C. Subjects maintained normal oral hygiene and dietary habits throughout the study. At the end of the 15-d treatment period, the enamel slabs were replaced and, after a one-week washout period, subjects repeated the experiment with another milk sample. This was repeated until each subejct had used all three milk samples.

Enamel sectioning and microradiography

Each treated enamel half-slab, paired with its untreated control half-slab, was embedded in methacrylate resin. Sections approximately 200 μm thick were cut from the embedded slabs perpendicular to the lesion surface through the midline of both half-lesions using an internal annulus saw microtome (Leica 1600, Leica, Germany). These sections were then lapped to produce 80±5 μm thick sections using a RotoPol/RotoForce 4 lapping instrument (Struers, Denmark) with 1200 and 2400 grit lapping paper. These sections of the remineralized half-lesion (treated) and the paired demineralized control half-lesion (untreated) were then radiographed along with an aluminium stepwedge of 10×14 μm thick increments. Microchrome High Resolution glass plates ($1 \times 3 \times 0{\cdot}06$ in., Microchrome, USA) were used along with nickel filtered copper Kα radiation at 20 kV, 10 mA for 5 min.

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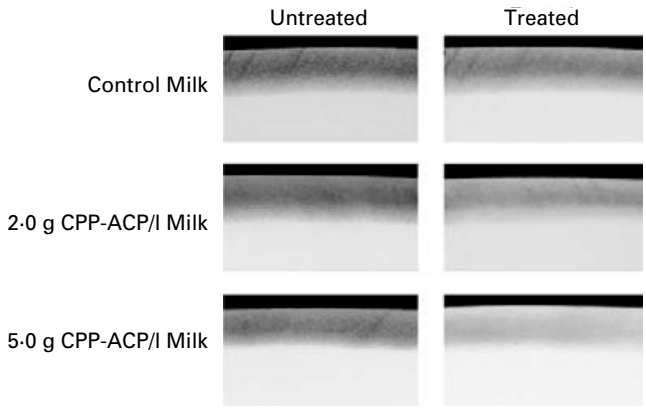

Fig. 1. Representative microradiographs of enamel lesions exposed to milk containing no added CPP-ACP, 2·0 and 5·0 g CPP-ACP/l, together with their control untreated lesions. The microradiographic images were taken of the sectioned enamel lesions. The top of the enamel section is at the top of each image. The dark zones on each image represent mineral loss. Treatment has resulted in an increase in mineral content.

Each glass plate was developed in 20 ml Microchrome Developer D5 (1 : 4 dilution, Microchrome, USA) for 4 min, then placed into glacial acetic acid stop bath for 30 s and then fixed in Microchrome Fixer F4 (1 : 4 dilution, Microchrome, USA) for 4 min.

Microdensitometry

Radiographic images of the lesions were viewed via transmitted light through a microscope and were acquired by a charge-coupled device digital camera (Spot Insight, Diagnostic Instruments, Inc., MI, USA). Images of the lesions and the neighbouring areas of sound enamel were scanned and analysed using the line luminance function of the imaging software Optimate (version 5.2). An area free of artifacts or cracks was selected for analysis, and a total of 200 readings taken from the tooth surface through the lesion to sound enamel were completed for each scan. The aluminum stepwedge image on each slide was also scanned and the averaged step gray value readings were plotted against aluminum thickness. The section thickness was measured and the % mineral data computed using the equation of Angmar (1963) and the linear absorption coefficients of aluminium, organic matter plus water and apatitic mineral. The image of the median strip of sound enamel between the two lesions was scanned six times and averaged to give a control sound-enamel densitometric profile. The lesion images [remineralisation (treated) window and demineralized (untreated) control window] to 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 % mineral profiles were determined.

Table 1. Remineralization (% R) values for enamel subsurface lesions exposed to control milk and milk containing 2·0 g and 5·0 g casein phosphopeptide-amorphous calcium phosphate (CPP-ACP)/l bovine milk in a human *in situ* model

Values are for 10 individual subjects

	Remineralization (% R)		
Subject No.	Control Milk	Milk with 2·0 g CPP-ACP/l	Milk with 5·0 g CPP-ACP/l
1	5·7	7·3	10·3
2	2·0	6·9	9·9
3	5·6	8·4	11·3
4	6·6	8·5	14·2
5	3·8	7·2	11·2
6	3·0	8·1	8·9
7	4·9	8·0	11·5
8	4·4	6·8	15·8
9	4·7	8·0	10·1
10	4·9	8·5	10·9
Mean	4·6[a]	7·8[a] (70%)[b]	11·4[a] (148%)[b]
S.E.M.	0·4	0·2	0·7

[a]All % Remineralization values significantly different to each other ($P<0·001$)
[b](Percentage increase in remineralization relative to control milk)

Data analysis

The vol % mineral profile of each untreated and treated lesion was compared with the vol % mineral profile for the median sound enamel of the same section (Reynolds, 1997a). Using trapezoidal integration, the difference between the area under the densitometric profile of the demineralized lesion (untreated) and the median sound enamel (ΔZd) and the difference in area under the densitometric profile of the remineralized lesion (treated) and the median sound enamel (ΔZr) were calculated. Percent remineralization (% R) was then calculated from the ΔZ values using the following equation:-

$$\% R = (\Delta Zd - \Delta Zr)/\Delta Zd \times 100$$

The differences between mean % R values obtained following exposure to the three milk samples were statistically analysed using an ANOVA for a completely randomized block design with a Tukey *post hoc* test (SPPS version 11).

Results

All three milk samples were shown to remineralize enamel subsurface lesions using this human *in situ* model. Representative microradiographs are shown in Fig. 1 and the mean remineralization (% R) values are shown in Table 1. Enamel lesions exposed to the two milk samples containing added CPP-ACP remineralized to a significantly greater extent than those exposed to the control milk.

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Exposure to milk containing 2·0 and 5·0 g CPP-ACP/l effectively caused a 70% and 148% increase in percent remineralisation, respectively, compared with that of the control milk. Statistical analysis showed that the mean % R values for lesions exposed to the two milk samples with added CPP-ACP were both significantly higher than the % R value following exposure to the control milk ($P<0·001$). Further, the % R following exposure to the milk containing 5·0 g CPP-ACP/l was significantly higher than that produced by milk with 2·0 g CPP-ACP/l ($P<0·001$).

Discussion

This study demonstrates that addition of CPP-ACP to milk significantly enhances the ability of the milk to remineralize enamel subsurface lesions *in situ*. Dairy products are the most recognized food group exhibiting anticaries properties (Harper et al. 1986, 1987; Krobicka et al. 1987; Silva et al. 1987; Reynolds, 1998). The components largely responsible for this anticariogenic activity have been identified as calcium and phosphate ions and casein, in particular casein phosphopeptides [reviewed in Reynolds (1998)]. The casein phosphopeptides (CPP) have been shown to stabilize amorphous calcium phosphate as nanoclusters of bioavailable ions which are capable of remineralizing enamel subsurface lesions (early forms of tooth decay) when delivered from a mouthwash or chewing gum (Shen et al. 2001; Reynolds et al. 2003).

Bovine milk has been shown to be anticariogenic in animal caries models (Reynolds & Johnson, 1981) and also to remineralize enamel subsurface lesions *in vitro* (McDougall, 1977; Mor & Rodda, 1983). Bovine milk contains around 30 mM-calcium where only approximately 10 mM is not bound in casein micelles and of that only approximately 2 mM is free calcium ions (Neville et al. 1994; Tanaka et al. 1999). The calcium in casein micelles is unlikely to be available to diffuse across a relatively intact enamel surface layer into a subsurface lesion and therefore milk may have limited ability to remineralize enamel lesions *in situ*. To our knowledge the current study is the first to demonstrate that bovine milk when consumed normally does have the ability to remineralize enamel subsurface lesions *in situ*. However, that ability was substantially enhanced by the addition of CPP-ACP. The addition of 2·0 g and 5·0 g CPP-ACP/l to normal milk adds an extra 7·1 and 17·8 mM-calcium, respectively. The substantial increase in enamel remineralization with only a relatively small amount of added calcium is consistent with the majority of the calcium in the casein micelle being unavailable and the superiority of the calcium ion bioavailability in the form of CPP-ACP.

The remineralization of enamel subsurface lesions by milk indicates that it is an excellent vehicle for the addition of CPP-ACP to further enhance that remineralization ability and substantially increase milk's anticariogenic properties. Milk should be superior to other CPP-ACP vehicles that do not have intrinsic remineralization potential.

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