**The Effect of Casein Phosphopeptide-Amorphous Calcium Phosphate on enamel surface rehardening. An in vitro study**

**ABSTRACT**

**Aim** Casein phosphopeptide stabilised amorphous calcium phosphate (CPP-ACP) has been shown to remineralise enamel subsurface lesions. The aim of this study was to determine the effect of a paste containing 10% CPP-ACP (Toothmousse; GC) on enamel surface microhardness in vitro.

**Materials and methods** Thirty enamel blocks (3x2 mm) were prepared for the study group from the buccal surface of extracted teeth. The microhardness of the enamel surface was determined for the specimens initially before artificial demineralisation. After demineralization the specimens were incubated in 10% CPP-ACP for 5 min. Artificial saliva was used as a remineralising solution for the control group.

**Results** The mean baseline surface microhardness analyses of enamel blocks were 318.61±25.75 and 262.49±26.82. The data show that after demineralisation the mean microhardness decline in the experimental groups (247.05 ± 38.31 and 186.92 ± 63.77). The results showed that 10% CPP-ACP (281.79 ± 26.32) was comparable to the control (252.27 ± 31.05) for remineralisation of enamel subsurface lesions in vitro. The highest surface microhardness recovery (%SMHR) was found for control, but the differences of % SMHR between 10% CPP-ACP and control was not statistically significant (p=0.805).

**Conclusion** From the results it can be concluded that CPP-ACP may have positive effects on enamel remineralisation.

**Introduction**

Studies have been carried out in the past to understand the processes involved in the demineralisation of enamel at the early stage; however, there is still little known about if and to what extent these early stages are reversible. The investigation of enamel remineralisation has been mainly concentrated on caries lesions [Finke et al., 2000; Ten Cate et al., 1978]. Other factors, such as the acquired pellicle and plaque bacteria, which have been shown to influence the overall remineralisation process [Zahradnik, 1979], however, cannot be sufficiently incorporated in an in vitro study [Lippert, 2004].

Dairy products have been shown to be anticariogenic in animal and human in situ caries models [Reynolds and Johnson, 1981; Harper et al., 1987]. The cheese extract significantly increased the level of calcium in the experimental plaque, and the protective effect was associated with depression of enamel demineralisation and/or enhancement of remineralisation [Silva et al., 1987].

A new remineralisation technology has emerged, which uses an amorphous form of calcium phosphate, stabilised by phosphopeptides from the milk protein casein. These peptides have a remarkable ability to stabilise calcium phosphate in solution as amorphous calcium phosphate. Casein phosphopeptides (CPP) containing the cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- have the remarkable ability to stabilise amorphous calcium phosphate (ACP) in a metastable solution [Shen et al., 2001]. At 1.0% w/v, CPP-stabilised 60 mmol/L CaCl2 and 36 mmol/L sodium phosphate at pH 7.0 through the formation of colloidal casein-phosphopeptide calcium-phosphate complexes (CPP-CP). CPP-CP solutions, when applied to the molar teeth of rats twice daily reduced caries activity of the animals with 1.0% CPP-CP producing a 55% reduction relative to the distilled water control [Reynolds et al., 1995]. CPP ACP nanocomplexes have demonstrated to have anticariogenic potential in laboratory, animal, and human in situ experiments [Reynolds et al., 1995; Reynolds, 1987; Reynolds, 1997; Reynolds, 1998; Reynolds et al., 1999]. Reynolds, using an in situ caries model, showed that exposure of inset-enamel plaque to solutions containing tryptic peptides of casein significantly reduced enamel subsurface demineralisation. The CPP have a remarkable ability to stabilise calcium phosphate in solution and substantially increase the level of calcium phosphate in dental
plaque [Reynolds, 1998]. The casein peptides were incorporated into the inset-enamel plaque and were associated with an increase in the plaque's content of calcium and phosphate. The tryptic peptides responsible for caseinate's anticariogenic activity were the calcium-phosphate-stabilising casein phosphopeptides [Reynolds et al., 1995].

The objective of this study was to analyse microhardness alteration and remineralisation of artificial subsurface enamel lesions after application of a gel containing casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP: Toothmousse; GC).

Materials and methods

Preparation of specimens for microhardness testing

Erupted permanent first and second molars were obtained from the Dental School, Marmara University. Thirty extracted molars were disinfected by storage in a 13% sodium hypochlorite solution for at least 24 h after their roots and pulps had been removed.

Crowns were sectioned from the roots. The 30 enamel blocks (3x2 mm) were prepared for two study groups from the buccal surface of each extracted teeth. Only intact enamel areas on the buccal surface were used in this study. The buccal side was sectioned with a diamond saw and enamel pieces were embedded in epoxy resin.

The top surface of each cylinder was ground flat and polished with water cooled carborundum discs and 1200 grit waterproof silicon carbide paper, thereby removing about 200 µm of enamel. The enamel samples were then stored in tap water prior to investigation.

Experimental design

To produce demineralised lesions the samples were stored in acidic hydroxyethylcellulose (HEC, pH 4.8) for three days, in accordance with the method of Amaechi et al. [1998]. After demineralisation 15 blocks were incubated in 10% CPP-ACP (Toothmousse; GC) for 5 min. Immediately afterwards, the enamel samples were carefully washed using tap water to remove any excess acid. The enamel samples were then stored in tap water prior to investigation. For the control, fifteen blocks were kept in a remineralising solutions (1.5 mM calcium, 0.9 mM phosphate, 150 mM of KCl in 0.1 M Tris buffer, pH 7.0) for 18 hours.

Surface microhardness analysis

Mineral changes in superficial enamel layers are directly related to microhardness alterations, i.e. remineralisation of enamel carious lesions is associated with an increase of enamel surface microhardness [Feagin F et al., 1969; White DJ et al., 1988].

Baseline microhardness (SMH): enamel surface microhardness was then determined for each specimen after demineralization (SMH1). After demineralisation each group was incubated in either 10% CPP-ACP (study group) or a remineralizing solution for (control group), and the enamel surface microhardness measured was after this phase (SMH2).

Five indentations, spaced by 100 µm and in different parts of the enamel, were made at baseline (SMH), after demineralization (SMH1), and after 10% CPP-ACP and in the control specimens (SMH2). A digital Micro Vickers Hardness Tester (Wilson Wolpert Europe BV, 401 MVD, Netherland) fitted with a Vickers diamond and a 200N load was used to make indentations in the enamel surface. The loaded diamond was allowed to rest on the surface for 15 s. The mean values of all five measurements at the three different times (baseline, after demineralisation and after respective treatments) were then compared and the percentage of surface microhardness recovery (SMHR) was calculated for study and control groups [Wiegand et al., 2005].

Statistical analysis

Data were computerised and analysed using SPSS 16.0 software. Student’s paired t-test was used to compare surface microhardness before and after the treatments and surface microhardness recovery (%SMHR) among treatments. Repeated measures analysis of variance was used to assess statistical differences.

Results

The mean initial enamel surface microhardness of the specimens ranged from 318.61 ± 25.75 to 262.49 ± 26.82 Vickers units when measured at baseline (SMH). The data show that after demineralisation the mean decline in microhardness in the different experimental groups was nearly equal (247.05± 38.31 and 186.92± 63.77). The demineralisation caused a significant amount of surface softening compared to baseline enamel samples. The surface hardness progressively decreased (p<0.0001).

The data show that after demineralisation the decline in microhardness in the control and CPP-ACP group was nearly equal. No statistically significant differences were found in the surface hardness of those enamel samples, which were only demineralised. The acid challenge of the enamel resulted in 76% reductions, and after the 10% CPP-ACP-application the lesions resulted in remineralisation were 114.06%.

Tem per cent CPP ACP was able to increase the surface hardness of the enamel. After the 10% CPP-ACP-application surface microhardness was 281.79 ± 26.32 (p=0.007). After remineralising (remineralising solution), surface microhardness of control specimens was 252.27±31.05 (p=0.002). Table 1 shows the
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Analyses of enamel blocks with regard to SMH. Statistically significant differences in the surface hardness were found when comparing the difference of the surface hardness between 10% CPP-ACP and control groups after treatments (Fig. 1) (p > 0.021). The results showed that the control group had higher surface rehardening of enamel subsurface lesions in vitro.

The mean and SD of %SMHR for 10% CPP-ACP was 81.09 ± 75.81 and for the control it was 101.49 ± 79.70. The highest surface microhardness recovery (%SMHR) was found for the control group. But the differences in %SMHR between 10% CPP-ACP and control was not statistically significant (p = 0.805) (Fig. 2).

The microhardness values imply increased protection of the enamel specimens with 10% CPP-ACP and control.

This study demonstrated that CPP-ACP significantly enhanced the remineralisation of enamel surface lesions in vitro.

Discussion

Remineralising agents and agents inhibiting the dissolution of apatite can prevent caries formation or arrest the progress of the caries lesions. This may be accomplished by adding or increasing calcium phosphate levels in plaque and saliva [Wiegand et al., 2005].

The demineralisation and remineralisation processes are difficult to detect at the early stages on enamel by visual inspection. There are many diagnostic techniques for detecting the demineralisation which occurred as a result of the caries process [Yaskell et al., 1998].

Several new approaches have been made with the investigation of demineralisation/remineralisation cycles. A major drawback of previous microhardness studies was that the remineralised enamel was not subject to subsequent demineralisation.

Mineral deposition takes place as crystal growth, rather than as mineralisation of the organic matrix in enamel. Furthermore, enamel remineralisation as surface precipitation has been suggested to be a seeded growth of hydroxyapatite-like material in which an amorphous precursor phase is formed that undergoes rapid transformation to crystalline hydroxyapatite. The exposure of surface softened enamel samples to a remineralisation solution resulted in the deposition of a fairly rough, crystalline surface layer [Lippert et al., 2004].

Demineralisation of the samples was performed according to the method of Amaechi et al. [1998] who showed that demineralisation with acidified hydroxyethylcellulose gel leads to superficial mineral loss with formation of a lesion beneath, thereby closely resembling an initial carious lesion. However, exposure to acidified hydroxyethylcellulose for only three days might produce caries-like lesions but also only a softened or demineralised enamel surface without the formation of a subsurface lesion and a surface layer [Amaechi et al., 1998]. However, microhardness alterations are directly associated with mineral changes in superficial layers. Kielbassa et al., 1999 found a clear relationship between microhardness and the mineral content of in-situ-induced enamel lesions.

Consequently, in the present study microhardness determination was performed to evaluate the capacity of 10% CPP-ACP to reharden demineralised enamel surface completely.

The CPP, by stabilising calcium phosphate in

### Table 1: Surface microhardness analysis of enamel blocks.

<table>
<thead>
<tr>
<th></th>
<th>SMH Initial (Mean±SD)</th>
<th>After Demineral. (Mean±SD)</th>
<th>After Treatm. (Mean ±SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPP-ACP</td>
<td>318.61±25.75</td>
<td>247.05±38.31</td>
<td>281.79±26.32</td>
<td>0.007</td>
</tr>
<tr>
<td>Control</td>
<td>262.49±26.82</td>
<td>186.92±63.77</td>
<td>252.27±31.05</td>
<td>0.002</td>
</tr>
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</table>

**FIG. 1** The mean hardness values for enamel samples, which were demineralised and subsequently remineralised with either CPP-ACP or control.

**FIG. 2** The percentage surface microhardness recovery (%SMHR) of CPP-ACP and control groups.
solution, can maintain high-concentration gradients of calcium and phosphate ions and ion pairs into the subsurface lesion, thus effecting high rates of enamel remineralisation at least equivalent to those obtained by constant-composition procedures.

For mineral deposition to occur within the body of the lesion, calcium and phosphate ions must first penetrate the surface layer of enamel. The relatively mineralised and charged nature of the surface layer results in restricted permeability of the ions and ion pairs necessary for mineral formation [Larsen et al., 1989].

Calcium phosphate stabilised by CPP to produce a metastable solution supersaturated with respect to the amorphous and crystalline calcium phosphate phases has been shown in this study to remineralise enamel subsurface lesions. The CPP-stabilised calcium phosphate solutions remineralised subsurface enamel lesions at a rate equal to or greater than those obtained with constant-composition procedures. By stabilising calcium phosphate in solution, the CPP can maintain high-concentration gradients of calcium and phosphate ions and ion pairs into the subsurface lesion, thus effecting high rates of enamel remineralisation [Reynolds, 1997; Wiegand et al., 2005].

The role of CPP-ACP has been described as localisation of ACP at the tooth surface which buffers the free calcium and phosphate ion activities, helping to maintain a state of supersaturation with respect to enamel depressing demineralisation and enhancing remineralisation [LeGeros, 1990].

The formation and transformation of different types of calcium phosphates related to remineralisation are either promoted or inhibited by the presence of various inorganic or organic elements [LeGeros, 1999].

CPP-ACP have been shown not only to stabilise amorphous calcium phosphate, but also to deliver and localise ACP at the tooth surface [Reynolds, 1998; Reynolds et al., 1999; Amaechi, 1998] and demonstrated that CPP could still be detected on the tooth surface 3h after consuming xylitol gum containing CPP-ACP [Reynolds et al., 2003]. Thereby, our results confirm those of Shen et al. [2001] who also showed that CPP-ACP remineralises enamel subsurface lesions in situ.

Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) exhibit anticariogenic potential in laboratory, animal, and human in situ experiments. The addition of CPP-ACP to either sorbitol- or xylitol-based gum resulted in a dose-related increase in enamel remineralisation, with 0.19, 10.0, 18.8, and 56.4 mg of CPP-ACP producing an increase in enamel remineralisation of 9, 63, 102, and 152%, respectively, relative to the control gum, independent of gum weight or type [Shen et al., 2001].

Remineralised enamel may be more resistant than normal tooth enamel to acid challenge, as normal enamel is calcium–deficient, carbonated apatite that has been found to be more soluble than hydroxyapatite, which has been attributed to differences in crystalinity and/or microstrain. The gum containing CPP-ACP produced approximately twice the level of remineralisation of enamel subsurface lesions in situ, with mineral that was more resistant to subsequent acid challenge, than the control sugar-free gum [Lijima et al., 2004].

The 2% CPP-ACP solution could significantly remineralise the artificial enamel subsurface lesion in vitro. The remineralising rates were increased with the experiment time (within 10 days) [Zhao and Cai, 2001].

Walker [2006] showed that the addition of 2.0-5.0 g CPP-ACP/l to milk substantially increases its ability to remineralise enamel subsurface lesions.

The complex formed is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), which has been shown to remineralise enamel subsurface lesions in vitro and in situ when delivered with a sugar-free chewing gum [Angmar-Mansson and ten Bosch, 1993].

CPP was found to stabilise high concentrations of calcium, phosphate and fluoride ions at all pH values (7.0-4.5). Remineralisation of the subsurface lesions was observed at all pH values tested with a maximum at pH 5.5 [Cochrane et al., 2008].

CPP-ACP at 0.5 to 1.0% produced a reduction in caries activity similar to that of a 500 ppm F solution and did not significantly affect the bacterial composition of fissure plaque. Since CPP-CP have been found to increase the levels of calcium and phosphate in plaque up to fivefold in human in situ caries models and short-term mouthwash studies [Reynolds, 1987; Reynolds, 1997]; the proposed mechanism of their anticariogenicity is that they act as a calcium-phosphate reservoir, buffering the activities of free calcium and phosphate ions in the plaque fluid helping to maintain a state of supersaturation with respect to enamel minerals, thereby depressing enamel demineralisation and enhancing remineralisation [Reynolds, 1997]. The results of this study show that 10% CPP-ACP was able to increase the surface hardness of enamel and remineralise subsurface lesions in human enamel in vitro; 10% CPP-ACP application to lesions resulted in remineralisation of 114.06%.

Kumar et al. [2008] showed that CPP-ACP containing Tooth Mousse remineralised initial enamel lesions and it showed a higher remineralising potential when applied as a topical coating after the use of a fluoridated toothpaste. The inorganic components contained in high concentrations in CPP-ACP acted to enhance remineralisation of the enamel structure [Yamaguchi et al., 2006]. As an additive to foods and oral care products, CPP-ACP has the potential to control dental caries. CPP-ACP may have an anti-caries protective effect, by suppressing demineralisation, enhancing remineralisation, or possibly a combination of both [Angmar-Mansson and ten Bosch, 1993].
Conclusion

From the results it can be concluded that 10% CPP-ACP have a positive effect on enamel remineralization. Further investigations are necessary to analyse the caries-preventive effects of 10% CPP-ACP on enamel. However, clinical studies are recommended to evaluate whether the results of this in vitro study are also confirmed in vivo. In this connection, investigation on the remineralisation of surface softened enamel in vivo, has been carried out and will be published elsewhere.

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References