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# In vitro dentine remineralization with a potential salivary phosphoprotein homologue

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ABSTRACT

*Objective:* Advantages of introducing a salivary phosphoprotein homologue under standardized *in vitro* conditions to simulate the mineral-stabilizing properties of saliva have been proposed. This study longitudinally investigates the effects of casein, incorporated as a potential salivary phosphoprotein homologue in artificial saliva (AS) solutions with/without fluoride (F) on *in vitro* dentine lesion remineralization.

*Design:* Thin sections of bovine root dentine were demineralized and allocated randomly into 6 groups (n = 18) having equivalent mineral loss (ΔZ) after transverse microradiography (TMR). The specimens were remineralized using AS solutions containing casein 0 μg/ml, F 0 ppm ($C_0$–$F_0$); casein 0 μg/ml, F 1 ppm ($C_0$–$F_1$); casein 10 μg/ml, F 0 ppm ($C_{10}$–$F_0$); casein 10 μg/ml, F 1 ppm ($C_{10}$–$F_1$); casein 100 μg/ml, F 0 ppm ($C_{100}$–$F_0$) or casein 100 μg/ml, F 1 ppm ($C_{100}$–$F_1$) for 28 days with TMR taken every 7 days.

*Results:* Surface mineral precipitation, evident in group $C_0$–$F_1$, was apparently inhibited in groups with casein incorporation. Repeated measures ANOVA with Bonferroni correction revealed higher ΔZ for non-F and non-casein groups than for their counterparts ($p < 0.001$). Subsequent multiple comparisons showed that mineral gain was higher ($p < 0.001$) with 10 μg/ml casein than with 100 μg/ml when F was present in the earlier stages of remineralization, with both groups achieving almost complete remineralization after 28 days.

*Conclusion:* Casein is a potential salivary phosphoprotein homologue that could be employed for *in vitro* dentine remineralization studies. Concentration related effects may be clinically significant and thus must be further examined.



## 1. Introduction

The recent shift of the global caries burden from children to adults associated with root caries (Kassebaum et al., 2015) emphasizes the need for a better understanding of the disease and treatment of dentine tissues. It is well known that the saliva and pellicle play an important role in modifying the caries process (Dawes et al., 2015). However, while numerous studies have examined the interaction between the enamel and pellicle (Fujikawa, Matsuyama, Uchiyama, Nakashima, & Ujie, 2008; Zahradnik, 1979), little is known about its effects on the dentine tissue where mechanisms are more complex due to its composition (Preston, Smith, & Higham, 2008).

Statherins, proline-rich proteins (PRPs) and histatins, referred to as pellicle-precursor proteins, are key salivary phosphoproteins in the mineral regulation processes (Cochrane, Cai, Huq, Burrow, & Reynolds, 2010; Huq, Cross, Ung, & Reynolds, 2005; Hay, 1995; Levine, 1993; Moreno, Varughese, & Hay, 1979). These proteins inhibit calcium phosphate crystal nucleation and also inhibit apatite surface precipitation through their adsorption to tooth surfaces (Hay, Carlson, Schluckebier, Moreno, & Schlesinger, 1987; Schwartz, Hay, & Schluckebier, 1992).

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Although *in vitro* oral environment simulation is critical for understanding different mineral regulation processes, current protocols often overlook these proteins. Fujikawa et al. (2008) demonstrated the differences observed in *in vitro* enamel remineralization when protein-free artificial saliva and macromolecule-containing saliva were used. They found that the presence of salivary proteins resulted in inhibition of mineral gain on the surface and better mineral recovery within the lesion body in the presence of fluoride (F), whereas artificial saliva and F alone mainly resulted in surface mineral deposition. Considering the limitations and difficulties of using saliva or saliva derivatives, we had earlier suggested the use of a salivary phosphoprotein homologue for *in vitro* mineral regulation studies. The introduction of a salivary phosphoprotein homologue of known composition that could mimic the mineral-regulating properties of saliva under standardized conditions has been proposed to be useful in providing models for further studying the role of salivary phosphoproteins in mineralization and for the development and testing of anticariogenic agents (Romero et al., 2015).

The results of that report suggested that casein, a milk phosphoprotein, when incorporated *in vitro* in artificial saliva at concentrations relatively similar to pellicle precursor proteins behaved similarly to salivary phosphoproteins by inhibiting apatite growth and deposition on sound enamel and dentine surfaces. Moreover, it also exhibited a concentration-dependent inhibition of hydroxyapatite growth consistent with the results of previous seeded crystal growth studies performed using human salivary phosphoproteins (Moreno et al., 1979; Tamaki, Tada, Morita, & Watanabe, 2002). Our adsorption isotherm experiment also showed an affinity constant for casein ($K = 17.2 \times 10^3$ ml/μmol) (Romero et al., 2015) that was within the range reported for PRPs ($K = 14.7 \times 10^3$–$26.7 \times 10^3$ ml/μmol) (Moreno, Kresak & Hay, 1982).

Previously, we focused on the adsorption behaviour of casein and its interaction with the hydroxyapatite surface (Romero et al., 2015). The present study longitudinally investigates the effects of casein modelling incorporated as a salivary phosphoprotein homologue on mineral regulation, specifically on dentine lesion remineralization. Saliva is composed of numerous proteins that have complex interactions, and using a single-protein model poses several limitations. However, the authors believe that the results of this study could potentially provide insights into possible interactions between dentine tissues and salivary phosphoproteins, as well as exogenous proteins introduced through various remineralization strategies. Moreover, it could impart information that could lead to the development of better multi-protein artificial saliva models for *in vitro* studies.

The better-known milk derivative, caseinphosphopeptide complexed with amorphous calcium phosphate (CPP-ACP), has been extensively studied for its application on remineralization as well as for its anti-demineralization effects (Cochrane & Reynolds, 2012; Nongonierma & FitzGerald, 2012; Reynolds, 2009). This study however is different in that it used whole casein incorporated in the artificial saliva (AS) solutions in low concentrations similar to those of salivary phosphoproteins and without any additional calcium and phosphate such as in CPP-ACP and in milk. This report is first to demonstrate the effects of casein as a potential salivary phosphoprotein homologue on *in vitro* dentine remineralization.

## 2. Materials and methods

### 2.1. Preparation of artificial dentine cari es lesions

The labial surfaces of the roots of freshly extracted bovine incisors were abraded and flattened using 800-grit silicon carbide (SiC) paper to completely remove the cementum and expose the dentine. A low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) was then used to section the roots labio-lingually to create 108 plano–parallel sections of 180–200 μm thickness. Pilot tests were conducted to determine the appropriate specimen thickness for this study. Dentine sections were collected from the region starting at least 1 mm from the cementoenamel junction up to the junction of the radicular middle and apical thirds, ensuring a remaining dentine thickness of at least 1.5 mm in all specimens.

Using our own modification of the single thin-section technique (Exterkate, Damen, & ten Cate, 1993), the specimens were completely coated with acid-resistant varnish (Revlon, New York, NY, USA) in entirety and left to dry for 24 h. Thereafter, an adhesive polyethylene sheet (Plate Seal, Mixell, Tokyo, Japan) of 0.07 mm thickness was used to cover both sides of the specimens as reinforcement against specimen deterioration caused by prolonged immersion in solutions. A 2-mm treatment window was then created by removing the excess film over the labial surfaces, polishing with 2000-grit SiC paper to expose the dentine and applying varnish on the periphery. Varnish was also applied around the edges of the adhesive film for better sealing. An illustration of the prepared specimen can be seen in Fig. 1. The adaptation of the coating materials used in this technique was confirmed under a stereomicroscope (Nikon SMZ1000; Nikon, Tokyo, Japan).

The specimens were then immersed in a demineralizing solution containing 2.2 mmol/l $CaCl_2$, 2.2 mmol/l $KH_2PO_4$, 50 mmol/l acetic acid and 0.02% $NaN_3$ (10 ml per specimen) at pH 5.3 and 37 °C under constant agitation for 9 days but without monitoring and maintaining the degree of saturation throughout the demineralization period. Transverse microradiography (TMR) was performed using the method described below. The specimens were randomly allocated into 6 groups (n = 18) such that the mean mineral loss (ΔZ) was equivalent for all groups. The collective mean baseline values for the different TMR parameters for all the specimens after demineralization were: $\Delta Z = 4745 \pm 522$ vol% μm; lesion depth (LD) = $218.3 \pm 12.9$ μm; and surface density (SD) = $16.1 \pm 2.0$ vol%. The methods for obtaining the values of these parameters are described in Section 2.3. The effect of the coating

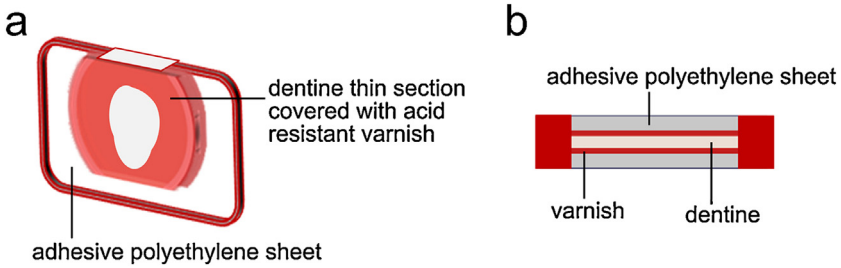


**Fig 1.** Schematic representation of the modified dentine single thin-section technique used in this study. (a) Thin dentine section covered in acid-resistant varnish and adhesive polyethylene sheet. (b) Coronal view of the specimen showing the different coating layers used in the specimen preparation.