DAIRY PRODUCTS AND DENTAL HEALTH

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Summary

Dental caries (tooth decay) is initiated via the demineralisation of tooth hard tissue by organic acids from the fermentation of dietary sugar by dental plaque odontopathogenic bacteria. Even though in most developed countries the prevalence of dental caries has decreased through the use of fluorides, the disease remains a major public health problem. Except for some reports associating nursing bottle caries with milk consumption, dairy products have been recognised for over 40 years as exhibiting an anticaries effect. Using laboratory, animal and human in situ caries models it has been shown that casein phosphopeptide amorphous calcium phosphate complexes (CPP-ACP) exhibit an anticariogenic activity. The casein phosphopeptides (CPP) are produced from a tryptic digest of the milk protein casein by aggregation with calcium phosphate and purification by ultrafiltration. The CPP have a remarkable ability to stabilise calcium phosphate in solution and substantially increase the level of calcium phosphate in dental plaque. Through their multiple phosphoseryl residues the CPP bind to clusters of amorphous calcium phosphate (ACP) in metastable solution, preventing their growth to the critical size required for nucleation and precipitation. The proposed mechanism of anticariogenicity for the CPP-ACP is that they localise ACP in dental plaque which buffers the free calcium and phosphate ion activities thereby helping to maintain a state of supersaturation with respect to tooth enamel. This depresses demineralisation and enhances remineralisation. The CPP-ACP, unlike fluoride, can be added to sugar-containing foods and therefore have commercial potential as an anti-cariogenic additive to foods and toothpastes.

I. DENTAL CARIES AND FLUOROSIS

Dental caries is initiated via the demineralisation of tooth hard tissue by organic acids from the fermentation of dietary sugar by dental plaque odontopathogenic bacteria (Loesche 1986). Even though in most developed countries the prevalence of dental caries has decreased through the use of fluorides, the disease remains a major public health problem (Carr 1987). In the recent Australian oral health survey 61% of 12 year olds examined showed signs of caries with a mean Decayed, Missing and Filled Tooth index (DMFT) of 1.8 (Barnard 1993). By the age 25-29 years, 98% of those examined were affected by caries with a mean DMFT of 11.7, which rose to 21.0, with a 6% decayed component, by 65 years of age. The estimated economic burden of treating dental caries in Australia in 1991 was $471 million, being higher than that for other dietary related diseases including coronary heart disease, hypertension or stroke (Crawley et al. 1992). In developing countries where the availability of industrialized food products is increasing caries prevalence is also increasing (Thylstrup and Fekersov 1988). Recent studies have highlighted a number of socio-demographic variables associated with caries risk; high risk being associated with ethnicity and low socio-economic status (Spencer et al. 1986). The level of high-risk individuals has remained constant even though the overall severity and prevalence of disease in the community has decreased (Wright 1986).

In countries where fluorides are used it is now recognised that the level of dental fluorosis has increased (Ekstrand et al. 1988). In recent surveys in the USA and Australia the prevalence of

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fluorosis was found to have increased in both fluoridated and non-fluoridated areas (Burt 1992; Riordan 1993a). From a recent Western Australian study, 48.4% of the seven to eight year old children examined exhibited fluorosis of their maxillary permanent central incisors (TF fluorosis classification scores of 1-3), with 43.5% of the children showing mild forms (TF scores 1-2) and 4.9% a more severe form (TF score 3) (Riordan 1993a). This represented a 100% increase in the TF score 3 prevalence that was found in a similar Western Australian study in 1991 (Riordan 1991). Fluorosed enamel is characterised by the formation of a more porous enamel with a subsurface hypomineralisation and can occur following either an acute or chronic exposure to above-threshold levels of fluoride during enamel formation (Ekstrand et al. 1988). Riordan (1993b) recently reported that TF scores of 2 or greater were easily noticed by lay observers and when the TF score was 3 the fluorosis aroused concern. The increased prevalence of dental fluorosis has been attributed to the inadvertent ingestion of fluoride toothpaste, infant formula consumption particularly when the formula has been reconstituted with fluoridated water, the use of fluoride supplements and a general increase in the fluoride level of infant foods and beverages through processing with fluoridated water (Riordan 1993a).

In a recent Australian consensus conference on the 'Appropriate Fluoride Exposure for Infants and Children' it was recommended that toothpaste manufacturers produce a 400 ppm fluoride paste specifically for children and that fluoride supplements be phased out in an approach to reduce the prevalence of fluorosis (Riordan 1993c). However, no data exists to demonstrate the anticariogenicity of the 400 ppm fluoride paste and a recent study by Koch et al. (1990) showed that a 250 ppm fluoride paste had a significantly lower anticaries efficacy than the normal 1000 ppm fluoride paste. If these measures to limit fluoride exposure are unsuccessful in lowering the prevalence of fluorosis in Australia then the next recommendation would be the reduction or elimination of fluoride in the reticulated water supply. It is possible therefore, that these measures implemented to reduce fluoride exposure may in fact produce an increase in caries prevalence particularly in the high-risk groups.

In conclusion, dental caries is still a major public health problem in Australia, particularly in ethnic and lower socio-economic groups and may become worse as measures are taken to reduce fluoride exposure. This highlights the requirement for the development of a non-toxic, anticariogenic agent that could be added to toothpaste, mouthwash and food in an approach to lower caries experience. It would be particularly useful if the anticariogenic agent was a natural food derivative as then it would be considerably easier to obtain the appropriate regulatory approval for the agent as a food additive. With approval as a food additive the agent could be incorporated into sugar-containing foods to target the high-caries-risk groups.

II. ANTICARIOGENICITY OF DAIRY PRODUCTS

Except for some reports associating nursing caries with milk consumption the food group most recognised as exhibiting anticaries activity is dairy products [milk, milk concentrates, powders and cheeses] (Reynolds and Johnson 1981; Rosen et al. 1984; Harper et al. 1986; Thylstrup and Fejerskov 1986; Harper et al. 1987; Krobicka et al. 1987; Silva et al. 1987). Using in vitro, animal and in situ caries models, the components largely responsible for this anticariogenic activity have been identified as casein, calcium and phosphate (Rosen et al. 1984; Harper et al. 1986; Harper et al. 1987; Krobicka et al. 1987; Silva et al. 1987; Schweigert et al. 1946a; Schweigert et al. 1946b; Shaw 1950; Bavetta and McClure 1957; Holloway et al. 1961; Reynolds and del Rio 1984; Reynolds and Black 1987a; Reynolds and Black 1987b; Reynolds 1987; Reynolds and Black 1989). The bovine milk phosphoprotein, casein, which is known to interact with calcium and phosphate (Reeves and Latour 1958) and is a natural food component is an obvious candidate for an anticariogenic food and toothpaste additive. In the early studies on the anticariogenicity of casein the insoluble acid form (casein HCl) was used which required very high levels for activity (Schweigert et al. 1946a; Schweigert et al. 1946b; Shaw 1950; Bavetta and McClure 1957).

More recently lower levels of soluble caseinate have been shown to be anticariogenic in a rat caries model when added to the drinking water (Reynolds and del Rio 1984) or as an ingredient or supplement in confectionery (Reynolds and Black 1987a; 1987b). The level of caseinate (17% w/w) which significantly reduced the cariogenicity of the chocolate confection was considered too high by the manufacturers due to the unpalatability of the caseinate. At a much lower, palatable level (2% w/w) the caseinate did not significantly change the confection's cariogenicity (Reynolds and Black 1989). It was concluded that casein's adverse organoleptic properties and large amount required for efficacy precluded its use as a food or toothpaste additive to lower the risk of caries.

(a) Anticariogenic casein phosphopeptides

Using a human intra-oral caries model it has been shown that digestion of caseinate with trypsin did not destroy the protein's ability to prevent enamel sub-surface demineralisation (Reynolds 1987). Tryptic peptides of casein were found incorporated into the intra-oral appliance plaque and were associated with a substantial increase in the plaque's content of calcium and phosphate. It was concluded that the tryptic peptides responsible for the anticariogenic activity were the calcium phosphate sequestering phosphopeptides. The major casein phosphopeptides (CPP) released by trypsin that sequester calcium phosphate are Bos α_{s1}-casein X-5P (f59-79) [1] and Bos ß-casein X-4P (f1-25) [2] together with smaller amounts of Bos α_{s2}-casein (f1/2-21) and Bos α_{s2}-casein (46-70)

[1] Gln-Met-Glu-Ala-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Gln-Lys. - α_{s1}(59-79).

[2] Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(*P*)-Leu-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-Ser-Ile-Thr-Arg. - ß(1-25).

The α_{s2}-casein peptides, α_{s2}(1-21) and α_{s2}(46-70) also contain the phosphoseryl cluster sequence -Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-. The CPP released by trypsin are 10% w/w of caseinate and through their multiple phosphoseryl residues sequester their own weight in calcium phosphate to form colloidal complexes (Reeves and Latour 1958). As the CPP are not associated with the unpalatability (Swaisgood 1982) or antigenicity (Ametani et al. 1987) of the caseins and have the potential for a specific anticariogenicity at least ten times greater on a weight basis, then their potential as a food and toothpaste additive is considerably better than that of the intact proteins. A simple and efficient purification procedure of the CPP has recently been developed involving ultrafiltration of calcium phosphate-induced complexes of the multiple phosphoseryl-containing peptides from a tryptic digest of casein (Reynolds 1992). The peptides produced by this procedure have been comprehensively characterised (Adamson et al. 1993; Reynolds et al. 1994). The major peptides of the preparation are ß(1-25) and α_{s1}(59-79) and its deamidated forms with minor amounts of α_{s2} peptides. All peptides contain the sequence Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu. The individual peptides of the preparation were identified by amino acid composition and sequence analyses after purification to homogeneity by anion exchange FPLC and reversed-phase HPLC (Reynolds et al. 1994). Prior to sequence analysis the labile phosphoseryl residues were converted to *S*-ethyl cysteinyl residues by ß-elimination (Reynolds et al. 1994).

(b) Interaction of CPP with calcium phosphate

The CPP have marked ability to stabilise calcium phosphate in solution. Using reversed-phase HPLC and anion exchange FPLC the major peptide of a CPP preparation, α_{s1}(59-79) has been purified and used to characterise its interaction with calcium phosphate. Solutions containing 0.1% w/v α_{s1}(59-79) at various pH, calcium and phosphate concentrations, but constant ionic strengths were allowed to attain equilibrium. Volumes of 10% or less of each equilibrated solution were centrifuged through a micropartition filter with a 2000 M_r exclusion limit. These filters do

not retain calcium phosphate ions or ion pairs. Calcium and inorganic phosphate (Pi) concentrations in the original solution (to confirm no precipitation) and the ultrafiltrate were determined and the peptide bound calcium phosphate calculated. The peptide α_{s1}(59-79) was found to maximally bind 24 Ca and 16 Pi per molecule. The ion activity products for the various calcium phosphate phases [hydroxyapatite (HA); octacalcium phosphate (OCP); tricalcium phosphate (TCP); amorphous calcium phospate (ACP); and dicalcium phosphate dihydrate (DCPD) were determined from the free calcium and phosphate concentrations at each pH using a modified computer program that calculates the ion activity coefficients through the use of the expanded Debye-Hückel equation and takes into account the ion pairs $CaHPO_4^0$, $CaH_2PO_4^+$, $CaPO_4^-$ and $CaOH^+$ the dissociation of H_3PO_4 and H_2O and the ionic strength. The only ion activity product that significantly correlated with calcium phosphate bound to the peptide independently of pH was that corresponding to ACP [$Ca_3(PO_4)_{1.87}(HPO_4)_{0.2}.xH_2O$] indicating that this is the phase stabilised by α_{s1}(59-79). In neutral and alkaline supersaturated calcium phosphate solutions ACP nuclei spontaneously form. The results therefore indicate that α_{s1}(59-79), through the multiple Ser(*P*) sequence Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu, bind to ACP clusters in metastable solutions preventing their growth to the critical size required for nucleation and precipitation. The binding of α_{s1}(59-79) to ACP results in the formation of colloidal complexes with the unit formula $[\alpha_{s1}(59\text{-}79)(ACP)_8]_n$ where n is equal to or greater than one. It is possible that the predominant form is n = 6 as α_{s1}(59-79) cross-linked with glutaraldehyde in the presence of ACP runs as a hexamer on polyacrylamide gel electrophoresis. Interestingly, the synthetic octapeptide AcGlu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-GluNHMe (Perich et al. 1992) only binds 12 Ca and 8 Pi per molecule i.e. $(ACP)_4$ indicating that the other residues and/or conformational specificity are essential for full ACP binding. Synthetic peptides corresponding to the *N*-terminal sequence Gln-Met-Glu-Ala-Glu and the *C*-terminal sequence Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Gln of α_{s1}(59-79) did not bind calcium phosphate. A 1.0% w/v CPP solution can stabilise 60 mM $CaCl_2$ and 36 mM sodium phosphate at pH 7.0 to form colloidal amorphous calcium phosphate-CPP complexes (CPP-ACP) with only 4.94 ± 0.46 mM free calcium and 4.90 ± 0.08 mM free phosphate. This solution has been studied using a variety of in vitro, human in situ and animal caries models.

(c) Anticariogenicity of CPP-ACP in the rat

The ability of casein-phosphopeptide amorphous calcium-phosphate complexes (CPP-ACP) to reduce caries activity was investigated using specific-pathogen-free rats orally infected with *Streptococcus sobrinus* 6715WT-13 (Reynolds et al. 1995). CPP-ACP solutions (100 µl) were applied to the animals molar teeth twice daily. Other groups of animals received 100 µl of solutions containing either 500 ppm F^-, the non-phosphorylated peptides of the casein tryptic digest or the synthetic octapeptide, Ac-Glu-Ser(*P*)-Ile-Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu-NHMe, corresponding to the common sequence in the major α_{s1}-and ß-peptides. The animals consumed a sucrose/gluten-based diet which did not contain dairy products. The CPP-ACP significantly reduced smooth surface caries activity in a dose response fashion with 0.1% w/v CPP-ACP producing a 14% reduction and 1.0% w/v CCP-CP a 55% reduction relative to the distilled water control. A similar, but slightly smaller reduction was found with fissure caries activity. CPP-ACP at 0.5-1.0% w/v produced a reduction in caries activity similar to that of 500 ppm F^-. The anticariogenicity of CPP-ACP and fluoride were additive as animals receiving 0.5% CPP-ACP plus 500 ppm F^- had significantly lower caries activity than those animals receiving either CPP-ACP or fluoride alone. The tryptic digest of casein with the phosphopeptides selectively removed showed no anticariogenic activity at 0.5% w/v. The non-phosphorylated casein peptides contained a similar level of arginyl residues to that of the casein phosphopeptides. The results therefore indicate that the anticariogenicity of the CPP-ACP is not associated with an arginine-based pH rise (Reynolds and Riley 1989). The synthetic octapeptide-calcium phosphate complex significantly reduced caries activity confirming that this calcium phosphate stabilising portion of the casein

phosphopeptides is associated with anticariogenicity. However, the lower molar specific activity of the synthetic octapeptide in binding ACP and in anticariogenicity compared with that of the longer α_{s1}- and ß-peptides suggests that other residues and/or conformational specificity are required for full activity. Other possible residues are those within the sequences -Ile-Val-Pro-Asn-Ser(*P*)-Val-Glu-Gln- and Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu- of the α_{s1}- and ß- peptides respectively. These sequences could have conformational specificity (ß-turn) required for full ACP binding and the hydrophobic residues could be involved as attachment anchors at hydrophobic sites in plaque and the oral mucosa.

(d) Anticariogenicity of CPP-ACP in human in situ studies

The ability of the 1.0% CPP, 60 mM $CaCl_2$ and 36 mM sodium phosphate pH 7.0 solution to prevent enamel demineralisation has been studied in a human in situ caries model (Reynolds 1991). The model has been described in detail previously (Reynolds 1987) and consists of a removable appliance containing a left and right pair of enamel slabs placed to produce a plaque retention site. The inter-enamel plaque that develops (3-5 mg) is bacteriologically similar to normal supragingival plaque (Reynolds 1987). On frequent exposure to sucrose solutions over a three week period the levels of mutans streptococci and lactobacilli increase and sub-surface enamel demineralisation results in the formation of an incipient 'caries-like' lesion. Two exposures of the CPP-ACP solution per day to the right pair of enamel slabs for 12 subjects produced a 51 ± 19% reduction in enamel mineral loss relative to the left-side, control enamel. The plaque exposed to the CPP-ACP solution contained 78 ± 22 µmol/g calcium, 52 ± 25 µmol/g P_i and 2.4 ± 0.7 mg CPP per g plaque dry weight compared with 32 ± 12 µmol/g calcium and 20 ± 11 µmol/g P_i in the control plaque. The level of the CPP was determined by competitive ELISA using an antibody that recognises both α_{s1}(59-79) and ß(1-25). Electron micrographs of immunoctochemically stained sections of the plaque revealed localisation of the peptide predominantly on the surface of microorganisms but also in the extracellular matrix. Although these results certainly indicate that CPP are incorporated into developing dental plaque the actual level determined by ELISA would not be a true representation of that incorporated due to the breakdown of the CPP in plaque through the action of phosphatase and peptidase activities (Reynolds 1987; Reynolds and Riley 1989). The incorporation of the CPP-ACP in the plaque resulted in a 144% increase in the plaque calcium and a 160% increase in plaque P_i with a Ca/P_i ratio consistent with ACP. These results have led to the proposition that the mechanism of anticariogenicity for the CPP-ACP is that they substantially increase the level of amorphous calcium phosphate in plaque depressing enamel demineralisation and enhancing remineralisation. In plaque, CPP-ACP would act as a resevoir of calcium and phosphate buffering the free calcium and phosphate ion activities thereby helping to maintain a state of supersaturation with respect to calcium phosphates. The binding of ACP to CPP is pH dependent with very little bound below pH 7.0.

(e) Remineralisation of enamel lesions by CPP-ACP

An in vitro model system (Dorr and Reynolds 1993), based on the system of Featherstone et al. (1986), has been used to study the effect of CPP-ACP solutions on remineralisation of artificial lesions in human third molars. The model involves the preparation of uniform, reproducible sub-surface enamel lesions which are cut into two with one half being used as the control to the other which is exposed to a remineralising solution. At the end of the treatment (usually ten exposures) the lesions are sectioned and subjected to microradiography and mineral content determined by microdensitometry. Using this system the 1.0% CPP-ACP solution used in the rat caries and in situ experiments replaced 56.3 ± 21.8% of mineral lost. A 0.1% CPP-ACP solution replaced 33.8 ± 18.9% of mineral lost.

A number of solutions containing various amounts of CPP (0.1-1.0%), calcium (6-60 mM) and phosphate (3.6-36 mM) at different pH values (7.0-9.0) have now been studied in this model system. The associations between the activities of the various calcium phosphate species in

solution and the rate of enamel lesion remineralisation for this series of solutions were then determined. The activities of the various calcium phosphate species in solution were calculated using the free calcium and phosphate concentrations, pH and an iterative computational procedure described above. The activity of the neutral ion species $CaHPO_4^O$ in the various remineralising solutions was found to be highly correlated with the rate of lesion remineralisation. The diffusion coefficient for the remineralisation process was estimated at 3×10^{-10} m^2s^{-1} and is consistent with the coefficients of diffusion for neutral molecules through a charged matrix. The rate of enamel remineralisation obtained with the 1.0% CPP-ACP solution was 3.3×10^{-2} mol HA/m^2/10 days which to our knowledge is the highest remineralisation rate ever obtained.

This finding that $CaHPO_4^O$ is the calcium phosphate species associated with enamel-lesion remineralisation is consistent with Gray's (1966) theory that the diffusion in and out of the enamel lesions is confined largely to neutral species. $CaHPO_4^O$ is the only neutral ion calcium phosphate species. $CaHPO_4^O$ after diffusion into the enamel lesion would dissociate and thereby increase the degree of saturation with respect to HA. The formation of HA in the lesion would led to the generation of H_3PO_4, which also being neutral would diffuse out of the lesion down a concentration gradient. The results indicate that the CPP-bound ACP, $CPP[Ca_3(PO_4)_{1.87}(HPO_4)_{0.2}.xH_2O]_8$ acts as a reservoir of the neutral ion species $CaHPO_4^O$ that is formed in the presence of acid. The acid can be generated by dental plaque bacteria; under these conditions, the CPP-bound ACP would buffer plaque pH by producing $CaHPO_4^O$. The increase in plaque $CaHPO_4^O$ would offset any fall in pH thereby preventing enamel demineralisation. Acid is also generated in plaque as H_3PO_4 by the formation of HA in the enamel lesion during remineralisation. This therefore could explain why the CPP-ACP solutions are such efficient remineralising solutions as they would consume the H_3PO_4 produced during enamel lesion remineralisation by generating more $CaHPO_4^O$ thus maintaining its concentration gradient into the lesion. These results are therefore consistent with the proposed anticariogenic mechanism of the CPP being the inhibition of enamel demineralisation and enhancement of remineralisation through the localisation of ACP at the tooth surface.

III. CONCLUSION

The anticariogenic potential of the CPP-ACP has been demonstrated in the rat caries model, the in situ human caries model and in vitro remineralisation models. The CPP-ACP and fluoride were shown to have additive effects in reducing caries experience suggesting that the CPP-ACP would have potential as a toothpaste additive to improve the efficacy of the current fluoride-containing dentifrices. Further, Australian dental health professionals are now recommending 400 ppm fluoride containing pastes for children under seven years of age due to the increasing prevalence of dental fluorosis. The CPP-ACP therefore may have an important role in toothpastes either alone or with 400 ppm fluoride for children in an approach to limit fluoride exposure without decreasing the efficacy of the paste. The proposed mechanism of the anticariogenicity for the CPP is that they stabilise and localise ACP at the tooth surface thereby buffering plaque pH and depressing enamel demineralisation and enhancing remineralisation. The CPP-ACP anticariogenic activity is greatest when the peptides are delivered at the same time as the cariogenic challenge and the CPP-ACP are a natural derivative of milk therefore unlike fluoride they could be added to sugar-containing foods. Preliminary results indicate that the CPP-ACP can be incorporated into confectionery without adverse organoleptic effects. The CPP-ACP therefore could have an important role as a food additive for the control of dental caries.

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