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An *in vitro* model of chlorhexidine-induced tooth staining

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Carpenter GH, Pramanik R, Proctor GB. An in vitro *model of chlorhexidine-induced tooth staining. J Periodont Res 2005; 40: 225–230. © Blackwell Munksgaard 2005*

Background: Tooth staining is a common feature of chlorhexidine treatment for periodontal disease and there is a large variation between patients as to the degree of their tooth staining. Although the mechanism of tooth staining is uncertain, diet, smoking and oral hygiene appear probable factors.

Objectives: This study investigated the role of saliva in chlorhexidine-induced tooth staining and used tea as the staining agent in an *in vitro* model with hydroxyapatite mimicking teeth.

Methods: Saliva has been used to create an acquired pellicle and in solution to mimic its effects *in vivo*. Using different combinations of tea, chlorhexidine and parotid saliva, substances binding to hydroxyapatite were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Using this system, tea, chlorhexidine and salivary proteins were clearly identifiable following staining by Coomassie Brilliant Blue.

Results: The results indicated that tea interacted with many salivary proteins, in particular proline-rich proteins and histatins. Chlorhexidine did not appear to complex with or precipitate salivary proteins nor prevent the formation of an acquired pellicle on the hydroxyapatite. In isolation, tea and chlorhexidine bound in small amounts to hydroxyapatite, but when added in combination, binding of both to hydroxyapatite was greatly increased. The acquired pellicle reduced chlorhexidine and tea binding, but conversely increased the binding of either tea or chlorhexidine alone to hydroxyapatite.

Conclusion: In conclusion, salivary proteins play an important role in the staining process and the combination of tea and chlorhexidine appears to be a very potent staining factor.

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Chlorhexidine is an antiseptic widely and successfully used to reduce or control plaque formation, although it has the major drawback of promoting tooth staining (1–3). The mechanism of extrinsic tooth staining appears to be varied and uncertain (4), although it is widely believed that polyphenols present in beverages such as tea and wine are a major cause [for a review see (2)]. Black tea contains two major groups of polyphenols – theaflavins and thearubicans, which give the tea its characteristic colour and flavour. Theaflavins form a small (2%) fraction of the dry weight of tea and are quite small structures (300–500 MW), whereas thearubigans form 10–15% of the dry weight and their structure is quite variable and generally much larger (approximately 3000 MW) (5). Polyphenols are secondary metabolites of plants and readily combine with proteins and can inactivate enzymes. They are therefore quite disruptive to digestion, although herbivores can overcome these effects by binding polyphenols with salivary proteins before they reach the gut (6). Initially it was thought that proline-rich proteins, which form up to 70% of total proteins in parotid saliva (7), were the high affinity binders of polyphenols because of their high proline content (8);

however, histatins (histidine-rich class of proteins, forming ~10% of total salivary proteins) also strongly bind polyphenols (9).

A subset of salivary proteins specifically binds to the surfaces of teeth to form what is called the acquired pellicle (10). The binding of proteins to teeth has been studied *in vivo* and *in vitro* using hydroxyapatite as a substitute for tooth enamel. Proteins most prominent in the hydroxyapatite pellicle include acidic proline-rich proteins, statherin, histatins and cystatins (11). Probably the most important role of the acquired pellicle is to maintain tooth integrity by reducing mineral loss and protecting teeth against abrasion. Thus the dental pellicle provides important functions; however, it may also mediate tooth staining by polyphenols found in tea and other dietary foods. In this study we have used an *in vitro* model in order to examine the influence of salivary proteins (both in solution and as an acquired pellicle) on chlorhexidine-induced staining of hydroxyapatite by tea.

Methods

Saliva collection

Stimulated parotid saliva was collected by means of a sterilized Lashley cup. The parotid (Stenson's) duct on the buccal mucosa was located just behind the third upper molar and the cup held in position by suction applied with the syringe. A sugar-free lemon drop was sucked ipsilateral to the cup and, after rejecting the first five drops, saliva was collected into pre-weighed tubes on ice.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Pre-cast 16% Tricine gels were run at 125 V, as per manufacturer's instructions (Invitrogen Ltd, Paisley, UK) for 90 min. Proteins in gels were visualized with 0.2% (w/v) Coomassie Brilliant Blue R250 (Sigma Ltd, Poole, Dorset, UK) in 25% methanol, 10% acetic acid at room temperature for 90 min and de-stained with 10% acetic acid for a further 90 min. Proteins were identified by their metachromatic staining for proline-rich proteins (12), molecular weight by comparison to standard proteins (Invitrogen Ltd) or by antibody staining as previously described (13).

Preparation of salivary acquired pellicle

Five milligrams of hydroxyapatite (crystalline form; Sigma) was added to 1 ml of fresh parotid saliva and the mixture was kept in suspension by rotation overnight at 4°C and centrifuged at 7000 *g* (6500 r.p.m.) for 15 min at 4°C. The residual saliva (unadsorbed protein fraction) was removed, stored on ice and the remaining sediment (salivary proteins adsorbed to the substrate, forming the pellicle) were washed three times with 1 ml Tris-buffered saline (0.1 M Tris, 0.15 M NaCl, pH 7.6). The residual saliva and protein-bound pellicle were then heated at 100°C with sodium dodecyl sulfate sample buffer (Invitrogen) for 3 min and analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or stored at −70°C for future use.

Preparation of tea solution

Extra Strong Tea (Marks & Spencer Co. Ltd, London, UK) was used at 2 g/100 ml double distilled water at room temperature. To prepare the tea, freshly boiled water was added then stirred for 3 min, left to settle and finally centrifuged (15 000 ***g*** for 5 min) and filtered to remove sediment.

Binding of salivary proteins to tea

Tea solution (1 µl, 10 µl and 50 µl) was added to parotid saliva (50 µl), made up to a final volume of 0.1 ml with ddH_2O and incubated at 37°C for 30 min. A sample was then taken for analysis by Tricine–polyacrylamide gel electrophoresis under non-reducing conditions and proteins visualized by Coomassie Brilliant Blue R250. For reducing conditions, the reducing agent dithiothreitol was added (0.5 mg/100 µl final volume) and samples boiled for 5 min.

In another experiment, parotid saliva (15 µl) was added to varying mixtures (3 µl) of chlorhexidine and tea, incubated at 37°C for 30 min and then analysed by Tricine–polyacrylamide gel electrophoresis.

Tea, chlorhexidine and salivary protein binding to hydroxyapatite

Mixtures of two or more components were incubated at 37°C for 30 min then clarified by centrifugation at 15 000 ***g*** for 10 min and the supernatant removed. Hydroxyapatite pellets were washed three times with Tris-buffered saline, then bound material eluted by boiling with sample sodium dodecyl sulfate buffer for 3 min for analysis by Tricine–polyacrylamide gel electrophoresis as per conditions described above.

Chlorhexidine was used as either a neat solution of a pre-prepared solution (Corsodyl, Brentwood, Middlesex, UK) or as a dilution of pure chlorhexidine (Sigma) to give an identical concentration (0.2% w/v) – both solutions produced similar results.

The following sequences of saliva, tea and chlorhexidine were bound to hydroxyapatite.

1. Hydroxyapatite treated with tea, chlorhexidine or a mixture of the two. To a 5 mg pellet of hydroxyapatite, 50 µl of either tea, chlorhexidine or 100 µl of a (1 : 1) mixture of chlorhexidine and tea were adsorbed onto hydroxyapatite by incubation at 37°C for 30 min with constant rotation. Tea/chlorhexidine was removed and pellets washed with Tris-buffered saline before analysis by Tricine–polyacrylamide gel electrophoresis.
2. Hydroxyapatite treated with parotid saliva, chlorhexidine or an equal mixture of the two. To three 5 mg pellets of hydroxyapatite 50 µl of either parotid saliva, chlorhexidine or a (1 : 1) mixture of the two were added and incubated together as above. Pellets were washed and analysed by Tricine–polyacrylamide gel electrophoresis. To another pellet, chlorhexidine was added and incubated as above. Excess chlorhexidine was removed and the pellet washed with Tris-buffered saline and then incubated with parotid saliva.

Following a second incubation period, the pellet was washed and analysed by Tricine–polyacrylamide gel electrophoresis. To a fifth pellet of hydroxyapatite, an initial incubation with parotid saliva was followed by chlorhexidine after removing excess parotid saliva.

Some hydroxyapatite pellets were dissolved by the addition of 10 µl concentrated HCl, which was neutralized by the addition of 30 µl of 1 M NaOH. Dissolved hydroxyapatite pellets were then analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as above.

All experiments were repeated three times with a separate gel for each experiment and levels of protein, tea and chlorhexidine were assessed by eye.

Results

Salivary proteins interaction

Parotid salivary proteins, in particular proline-rich proteins and histatins, interact with tea. Using Tricine–polyacrylamide gel electrophoresis with Coomassie Brilliant Blue (R250) staining and destaining in 10% acetic acid, parotid salivary proline-rich proteins stained metachromatically (pink), allowing their discrimination from blue-staining proteins such as histatins and amylase. Using this same system, a sample of tea stained as a wide smear and chlorhexidine as a low molecular weight spot. As shown previously (14), the interaction of tea and salivary proteins can be demonstrated by non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis as a reduction in staining of proteins (Fig. 1). Although chlorhexidine by itself did not have the same effect, it did accentuate the interaction of tea with saliva, as a greater number of proteins appeared to stain less intensely compared to tea plus parotid saliva. Under reducing conditions, all parotid salivary proteins appeared in Coomassie Brilliant Blue R250 stained gels (results not shown), suggesting no proteolytic degradation occurred.

Binding to hydroxyapatite

In this study, incubation of hydroxyapatite with chlorhexidine and tea solutions was used as a model for tooth staining. Following the incubation period, hydroxyapatite pellets were either analysed directly by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or were dissolved in acid before analysis; similar results were obtained by either method. Using the distinctive stain patterns of tea and chlorhexidine (Fig. 1) as a guide, detectable amounts of each were seen to bind to hydroxyapatite (Fig. 2, lanes 1 and 3). However, when chlorhexidine and tea were incubated together with hydroxyapatite, there was a considerable increase in tea and chlorhexidine binding to the hydroxyapatite pellet.

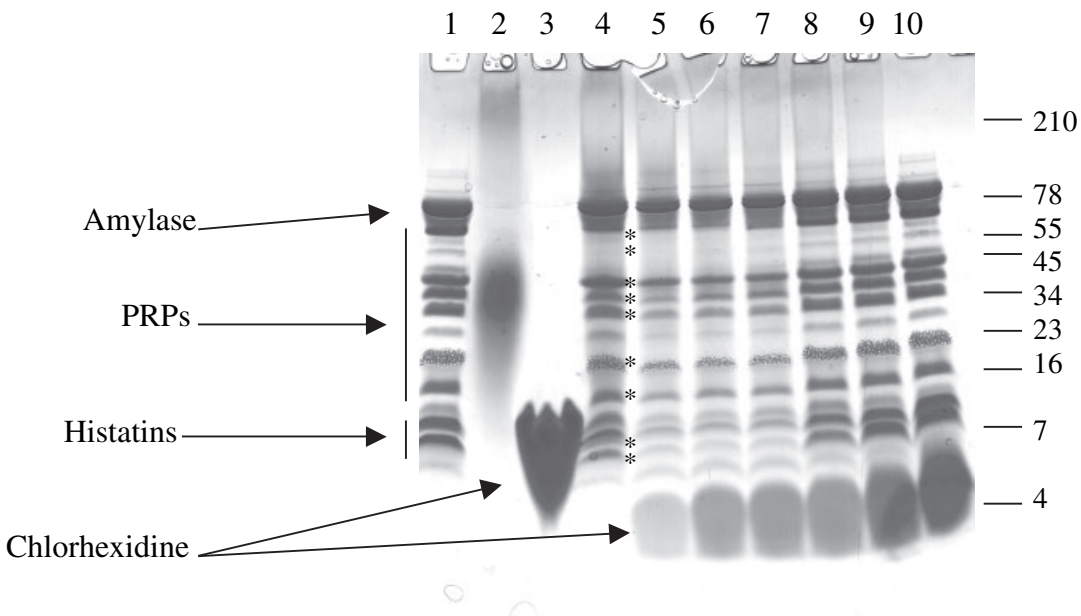

Fig. 1. Interaction of parotid salivary proteins with tea and/or chlorhexidine is indicated by reduced protein staining (*). Lane 1, 15 µl parotid saliva; lane 2, 15 µl 2% (w/v) tea solution; lane 3, 15 µl of 0.2% (w/v) chlorhexidine solution; lane 4, parotid saliva plus tea (15 + 3 µl); lanes 5–9, parotid saliva (15 µl) with 3 µl of a varying ratio of tea and chlorhexidine (lane 5, 20% chlorhexidine : 80% tea; lane 6, 40% chlorhexidine : 60% tea; lane 7, 50% chlorhexidine : 50% tea; lane 8, 60% chlorhexidine : 40% tea; lane 9, 80% chlorhexidine : 20% tea); lane 10, parotid saliva plus chlorhexidine (15 + 3 µl). Following incubation, samples were separated by non-reducing Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This gel is representative of three repeats of the same experiment. At the concentrations used, chlorhexidine by itself did not interact or precipitate any salivary proteins (compare lane 10 to lane 1). However tea did reduce the staining of several proteins (indicated by asterisks), which was accentuated by chlorhexidine (compare lanes 5–7 with lane 4). Figures on the right indicate the approximate positions of molecular weight markers (kDa). PRP, proline-rich protein.

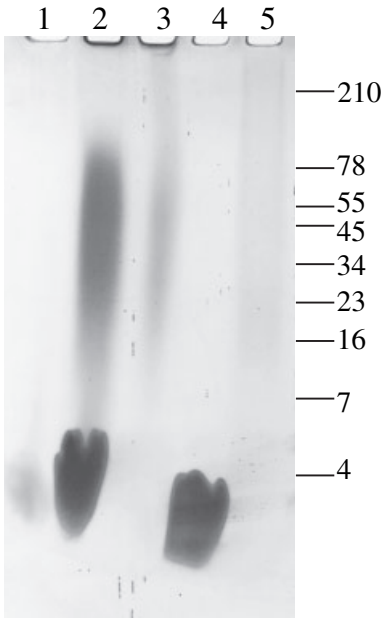

Fig. 2. Tea and chlorhexidine binding to hydroxyapatite. Lane 1, chlorhexidine eluted from hydroxyapatite pellet; lane 2, chlorhexidine and tea eluted from hydroxyapatite following simultaneous incubation; lane 3, tea eluted from hydroxyapatite; lane 4, 10 µl of chlorhexidine solution (0.2%w/v); lane 5, 10 µl of 2% (w/v) tea solution. Following incubation of tea, chlorhexidine or a 1 : 1 mixture of tea and chlorhexidine with hydroxyapatite, the pellet was washed with Tris-buffered saline, sodium dodecyl sulfate sample buffer added and loaded onto a Tricine gel. This gel is representative of three repeats of the same experiment. Following Coomassie Blue staining, the amount of tea (lane 3) or chlorhexidine (lane 1) binding to hydroxyapatite was less than if a comparable amount of chlorhexidine and tea were incubated together with hydroxyapatite (lane 2). Figures on the right indicate the approximate positions of molecular weight markers (kDa).

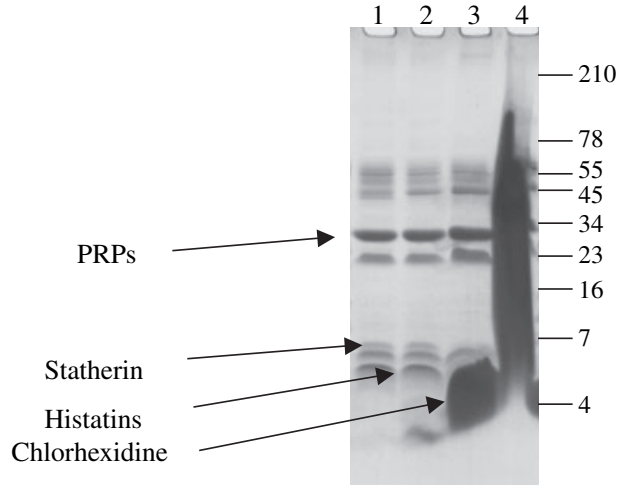

Fig. 3. The effect of a salivary pellicle on hydroxyapatite binding of chlorhexidine. Lane 1, parotid saliva incubated with hydroxyapatite to form an acquired pellicle; lane 2, hydroxyapatite pre-treated with chlorhexidine then incubated with parotid saliva; lane 3, hydroxyapatite pre-treated with parotid saliva then incubated with chlorhexidine; lane 4, simultaneous incubation of hydroxyapatite with a 1 : 1 mixture of parotid saliva and chlorhexidine. This gel is representative of three repeats of the same experiment. Chlorhexidine does not affect the formation of a salivary pellicle but parotid saliva increased chlorhexidine binding if present in solution or as an acquired pellicle. Figures on the right indicate the positions of molecular weight markers (kDa). PRP, proline-rich protein.

The effects of salivary pellicle

At the concentrations of hydroxyapatite and parotid saliva used, a subset of proteins bound (Fig. 3). These included acidic proline-rich proteins, statherin and histatins, as well as some other minor unidentified proteins. When the hydroxyapatite was pre-treated with chlorhexidine prior to saliva, there was no change in profile of proteins binding. However, chlorhexidine bound in larger amounts to hydroxyapatite if a salivary pellicle was present (compare Fig. 3, lane 2 with lane 3). Furthermore, chlorhexidine incubation with hydroxyapatite pre-treated with saliva did not appear to remove any protein from the hydroxyapatite, because the protein staining intensity was not reduced as assessed by eye. Incubation of both chlorhexidine and saliva with hydroxyapatite simultaneously caused the greatest amount of chlorhexidine binding (Fig. 3, lane 4). Due to the large amounts of chlorhexidine binding, it was unclear whether salivary protein binding to hydroxyapatite was altered. Repeated experiments using lesser amounts indicated an increase in the amount of salivary protein binding to hydroxyapatite in the presence of chlorhexidine (results not shown).

In further experiments, the interaction of tea, saliva and chlorhexidine with hydroxyapatite was studied. As before, the combination of tea and chlorhexidine caused greater amounts of tea and chlorhexidine binding to untreated hydroxyapatite. If the hydroxyapatite was pre-treated with parotid saliva to form an acquired dental pellicle, tea binding appeared greatest with tea only treatment rather than chlorhexidine and tea treatment (compare Fig. 4, lanes 4 and 5). However chlorhexidine binding to hydroxyapatite was still greatest when in combination with tea, and the salivary pellicle still increased chlorhexidine binding compared to chlorhexidine bindng to hydroxyapatite when no pellicle was present.

Discussion

The interaction of salivary proteins with tea was shown in this study by their reduced protein staining when resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained by Coomassie Blue. This method assumes that, following the incubation of tea and salivary proteins, complexes form that are too large to be resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing conditions. Using reducing conditions, a normal profile of salivary proteins was seen (data not shown), suggesting that complexes are dissolved. Following the addition of just 2% (w/v) tea solution to parotid saliva, a number of protein–tea

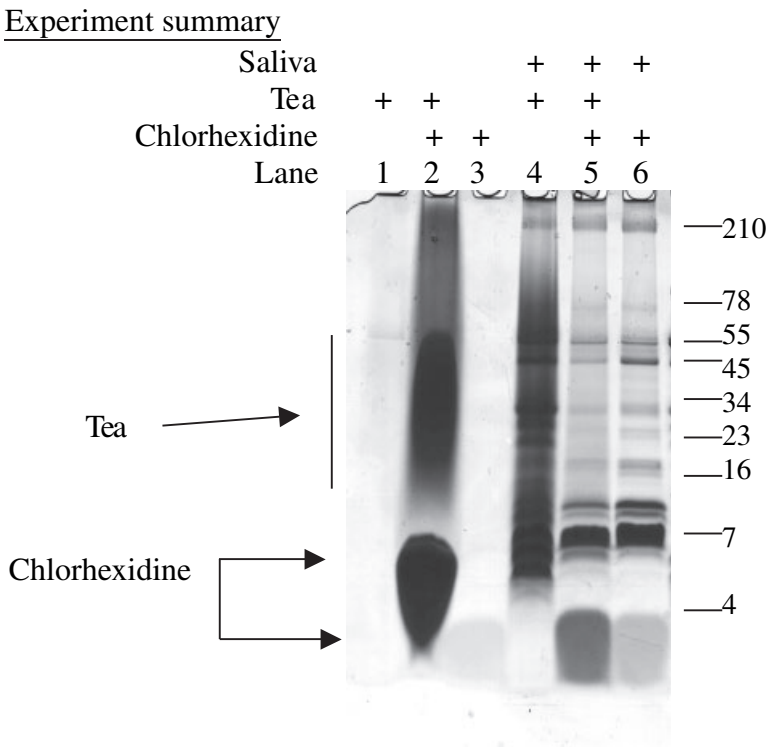

Fig. 4. The effect of a salivary pellicle on the interaction of chlorhexidine and tea with hydroxyapatite. Hydroxyapatite pellets (5 mg), some of which were pre-treated with parotid saliva to form an acquired pellicle (lanes 4–6), were incubated with chlorhexidine, tea or a mixture of tea and chlorhexidine. Following incubation, proteins in the samples were separated by Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained by Coomassie Brilliant Blue. Lane 1, tea eluting from hydroxyapatite; lane 2, tea and chlorhexidine eluting from hydroxyapatite; lane 3, chlorhexidine eluting from hydroxyapatite. Lanes 4–6, same order as lanes 1–3 except hydroxyapatite pellets had been pre-treated with parotid saliva. This gel is representative of three repeats of the same experiment. Figures on the right indicate the approximate positions of molecular weight markers (kDa).

interactions were detected using this method. This study, in agreement with other previous studies of salivary protein interactions with polyphenols (15, 16), showed that proline-rich proteins and histatins interacted with tea to a greater extent than other proteins such as amylase. Proteolytic degradation did not occur to any significant degree, as under reducing conditions the same samples resolved normally and there appeared to be no reduction in band intensity compared to a sample kept on ice.

The interaction of salivary proteins with tannins has been demonstrated previously [for a review see (6)], often using pure tannin or tannic acid. To indicate which salivary proteins had interacted with tannin, the insoluble precipitate formed was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15). The present study has used lower amounts of tannin (in tea) and at the concentrations of tea and saliva used, no visible precipitate was seen even after extensive centrifugation (15 min, 15000 *g*; results not shown), suggesting that soluble complexes were formed, as previously indicated (8), rather than larger insoluble complexes.

Hydroxyapatite has been used before as a substitute for human dental enamel in studies of human salivary proteins binding to form the acquired dental pellicle (17, 18). In our own studies, a similar profile of parotid salivary proteins bound to powdered bovine dental enamel as hydroxyapatite (14), suggesting that it forms a comparable substrate. A particular subset of parotid salivary proteins bound to hydroxyapatite, including acidic proline-rich proteins, statherin, histatins and some other unidentified proteins. From our experiments, it also appears that hydroxyapatite can bind chlorhexidine and tea, which both have distinctive stain patterns when analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. There was a substantial increase in the binding of both when added in combination to hydroxyapatite, which might not have been noticed in other *in vitro* models in which a water rinse step was used between cycles of chlorhexidine and tea (3). The substantially greater amount of tea binding to hydroxyapatite when incubated with chlorhexidine represents a possible mechanism for the increased tooth staining seen clinically.

Excessive tooth staining with chlorhexidine treatment is not universal in all patients (2), and one variable may be the amounts of salivary proteins. As well as interacting with polyphenols in solution, parotid salivary proteins also interact with hydroxyapatite to form an acquired dental pellicle. Histatins are notable in binding both polyphenols and hydroxyapatite and thus increased adsorption of histatins to a tooth surface could mediate greater staining. Our *in vitro* model suggested that chlorhexidine, when incubated in combination with saliva, did increase protein binding to hydroxyapatite (results not shown) and this may in turn mediate greater polyphenol interactions and increased tooth staining. Contrary to this, however, are the diverse effects of a salivary pellicle on tea binding. In isolation, tea binding was increased if a salivary pellicle was present. However, when tea was used in combination with chlorhexidine, a salivary pellicle reduced tea binding (although it was still greater than tea binding to hydroxyapatite without a salivary pellicle). Thus one variable in the degree of tooth staining seen between patients could be the amount and composition of the salivary pellicle. However, pellicles formed from whole mouth saliva suggest that there are not large differences in the salivary pellicle formation (18). Thus, although mediating an

effect on tooth staining, the salivary pellicle is unlikely to account for the large variations seen between different users of chlorhexidine.

The exact mechanism of stain promotion by chlorhexidine is unknown, although three possible mechanisms were reviewed by Watts and Addy (2):

1 chlorhexidine may accelerate the nonenzymatic browning reaction of protein and carbohydrate in the acquired pellicle;
2 chlorhexidine might denature components within the dental pellicle to accelerate formation of pigmented sulphides of tin and iron;
3 chlorhexidine could precipitate dietary chromagens.

From the results presented in this study using an *in vitro* model, we would suggest another mechanism might be that chlorhexidine accelerates formation of acquire pellicle and the accumulation of tea and chlorhexidine itself onto tooth surfaces. The dramatic increases seen in chlorhexidine and tea binding when added in combination to hydroxyapatite suggests that chlorhexidine may be modifying the polyphenols or their environment to increase binding of polyphenols (found in tea) onto dental surfaces. A study into the mechanism of the plaque-controlling action of chlorhexidine suggested it was most effective acutely and not as a result of a slowly released reservoir (19). A possible treatment strategy to avoid increased tooth staining could be to rinse the mouth with water shortly after the chlorhexidine treatment and certainly before eating or drinking (e.g. tea), thus allowing chlorhexidine to take effect without allowing the combination that caused the greatest amount of tea to bind hydroxyapatite in our *in vitro* model.

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