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Effect of gels containing chlorhexidine or epigallocatechin-3-gallate on the protein composition of the acquired enamel pellicle

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ABSTRACT

Objective: This study evaluated changes in protein profile of the acquired enamel pellicle (AEP) formed *in vivo*, after application of gels containing chlorhexidine or EGCG and further challenge with citric acid.
Design: AEP was formed in 9 volunteers for 2 h and then treated with one of the following gels: placebo, 400 µM EGCG or 0.012% chlorhexidine. A thin layer of gel was applied and after 1 min the excess was removed. One hour after gel application, the AEP was collected from the buccal surface (upper and lower jaw) of one of the sides with filter paper dipped in 3% citric acid. On the other side, erosive challenge was performed through gentle application of 1% citric acid (pH 2.5) for 20 s (using a pipette) followed by washing with deionized water. The AEP was collected as mentioned before. Proteomic analysis was performed through liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS). The MS/MS spectra obtained were compared with human protein databases (SWISS-PROT). Label-free quantitation was done using the PLGS software.
Results: In total, 223 proteins were identified. After treatment with EGCG and CHX gels, proteins with potential functions to protect against caries and erosion such as PRPs, calcium-bind proteins and *Statherin* were increased. When EGCG and CHX-treated AEPs were challenged with citric acid, there was increase in cystatins and *Profilin-1*.
Conclusion: CHX- and EGCG-treated AEPs, submitted to challenge with citric acid or not, had remarkable changes in their proteomic profiles.

1. Introduction

The acquired enamel pellicle (AEP) is an organic layer, free of bacteria, formed on the enamel surface by selective adsorption of proteins (mainly from salivary origin). It also contains lipids and glycoproteins, but at lower concentrations (Dawes, Jenkins, & Tongue, 1963). The presence of these organic components in the AEP confers important functions to this integument, such as lubrication and protection of the dental surface. The AEP acts as a physical barrier that reduces the dissolution of the hydroxyapatite thus protecting the tooth surface against acidic attacks (Buzalaf, Hannas, & Kato, 2012; Vukosavljevic, Custodio, Buzalaf, Hara, & Siqueira, 2014).

The efficacy of the AEP against acidic attacks depends on its composition and physical properties (Vukosavljevic et al., 2014, such as thickness (Amaechi, Higham, Edgar, & Milosevic, 1999). Regarding the composition, it has been reported that exposure to acids removes the globular outer layer of the pellicle, leaving the basal layer intact (Hannig & Joiner, 2006). Several proteins present in the AEP have the potential to regulate mineral homeostasis by inhibiting demineralization (Siqueira, Custodio, & McDonald, 2012), such as histatins (Siqueira, Margolis, Helmerhorst, Mendes, & Oppenheim, 2010), mucins (Cheaib, & Lussi, 2011; Nieuw Amerongen, Oderkerk, & Driessen, 1987), statherin and proline-rich proteins (PRPs) (Siqueira, Zhang, Helmerhorst, Gygi, & Oppenheim, 2007). Most of these proteins have been recently identified as resistant to removal by erosive or cariogenic challenges using proteomics tools (Delecrode, Siqueira, Zaidan, Bellini, Leite et al., 2015; Delecrode, Siqueira, Zaidan, Bellini, Moffa et al., 2015). These findings suggest that they might protect against caries and erosion.

Purified polyphenols such as epigallocatechin-3-gallate (EGCG), components from black tea, theaflavin and red wine are able to promptly adsorb onto the pellicle. This adsorption reduces the degree of pellicle elution by different solutions such as phosphate buffer and sodium dodecyl sulfate (Joiner, Muller, Elofsson, & Arnebrant, 2004;

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Joiner, Muller, Elofsson, Malmsten, & Arnebrant, 2003). When chlorhexidine is adsorbed onto the pellicle, the adsorption of salivary and black tea components is intensified (Joiner, Elofsson, & Arnebrant, 2006). Thus, in principle, chlorhexidine and polyphenols seem to be able to increase the protective role of the acquired pellicle against acidic attacks. Recently, it was reported that gels containing chlorhexidine or EGCG were able to fully prevent dentin erosion (Kato, Leite, Hannas, & Buzalaf, 2010), which was attributed to their ability to inhibit proteases that degrade the demineralized organic matrix (Kato et al., 2012). However, the modification of the AEP could also, at least partially, have been responsible for this effect, which should be evaluated.

Thus, the aim of this study was to detect changes in the protein profile of AEP *in vivo*, after application of gels containing chlorhexidine or EGCG and further challenge with citric acid. The null hypotheses tested were: (a) there is no difference in the protein profile of AEP after application of gels containing chlorhexidine or EGCG and (b) further challenge with citric acid does not alter the protein profile of these pellicles.

2. Materials and methods

2.1. Ethical aspects and human subjects

The study was performed following the guidelines of good clinical practice and conformed to the Declaration of Helsinki. Ethical approval for the study involving human subjects was granted by the Ethics Committee of the Bauru School of Dentistry, University of São Paulo (number of protocol 1.352.161; CAAE: 26930114.1.000.5414). Based on previous *in vivo* studies (Delecrode, Siqueira, Zaidan, Bellini, Moffa et al., 2015; Zimmerman et al., 2013) nine healthy adults (19–45 years old) were enrolled according to the study inclusion and exclusion criteria. Inclusion criteria were as follows: good oral health (i.e. no caries lesions or significant gingivitis/periodontitis), stimulated physiological salivary flow rate of > 1 mL/min; non-stimulated physiological salivary flow rate of > 0.1 mL/min; salivary pH between 6.8 and 7.2 (unstimulated flow). The average of unstimulated and stimulated salivary flow obtained from the volunteers was 0.38 and 1.05 mL/min, respectively and the mean of salivary pH was 6.99. Exclusion criteria were: pregnancy or breastfeeding; use of fixed or removable orthodontic appliances; use of medications that may alter salivary flow.

2.2. Acquired enamel pellicle formation, treatment and collection

AEP material was collected in the morning period to avoid circadian effects on salivary composition (Zimmerman et al., 2013). The volunteers were subjected to dental prophylaxis to remove the existing AEP. Then the pellicle was formed during 2 h. After pellicle formation, one of the gels was applied on the tooth surfaces (from 1st molar to 1st molar, both in maxillary and mandibular teeth) following a cross-over design. The gels used were: placebo gel (without active ingredients), gel containing 400 μm EGCG or gel containing 0.012% chlorhexidine. These gels were custom made and had exactly the same composition (hydroxyethylcellulose, propylene glycol, methylparaben, imidazolidinyl urea and water), except for the presence of chlorhexidine or EGCG or not (Kato et al., 2010). The concentrations of chlorhexidine and EGCG were chosen because in a previous *in situ* study these gels were able to completely prevent dentin erosion (Kato et al., 2010). The gels were applied with cotton swab for 1 min and then the excess was removed with a clean cotton swab.

After application of the gels, additional pellicle was allowed to form for 1 h (Joiner et al., 2006) before collection. AEP was collected from one of the sizes of the mouth, from 1st molar to central incisor (both from upper and lower jaw). The teeth were isolated with cotton rolls, washed with deionized water and dried by air. Two collection strips of 0.5 × 1.0 cm (electrode filter paper, Bio-Rad, Hercules, CA) presoaked in 3% citric acid were used to collect the AEP (one for the upper hemi arch and the other for the lower one) (Siqueira, Zhang, Helmerhorst, Gygi, & Oppenheim, 2007). To avoid contamination by the crevicular fluid, pellicle was not collected from the gingival third.

Pellicle formed on the teeth from the other side of the mouth was submitted to an erosive challenge, by pipetting 50 μL of 1% citric acid (pH 2.5) for 20 s, followed by thorough washing with deionized water. AEP was collected again from these sites and a pool was made, as described above. These procedures were repeated for two consecutive days for each treatment to provide enough material for proteomic analysis (Delecrode, Siqueira, Zaidan, Bellini, Moffa et al., 2015). At the end of each collection, volunteers rinsed with fluoridated solution (Colgate Plax Soft Mint) for 1 min to minimize the damage caused by the erosive challenge. The harvested material was placed in a 1.5 mL polypropylene microcentrifuge tube and stored at −80° C until proteomic analysis.

2.3. Preparation of the AEP samples

Samples were prepared for proteomic analysis as previously described (Ventura, Cassiano, Silva, Taira, Leite et al., 2017). The microcentrifuge tubes containing the filter papers were removed from the −80° C freezer. After defrost, the papers were cut into small pieces with the aid of sterile scissors and tweezers. These small pieces obtained from each of the 9 volunteers, in the two days, for each treatment, were pooled for proteomic analysis (Delecrode, Siqueira, Zaidan, Bellini, Moffa et al., 2015). This pool is necessary because it is not possible to obtain enough material for individual analysis.

To each pool, a solution containing 6 M urea and 2 M thiourea in 50 mM NH_4HCO_3 pH 7.8 was added (the volumes ranged between 2.2 to 2.6 mL, in order to cover completely the pieces of paper with the solution). The samples were vortexed for 10 min at 4 °C, sonicated for 5 min and centrifuged at 14,000*g* for 10 min at 4 °C. The supernatant was collected and this step was repeated once more, with the addition 0.4 mL of the above-mentioned solution on the cut papers for an adequate sample recovery. Recovered supernatants were stored together.

Then the filter papers were placed in tubes (Corning Costar® Spin-X® Plastic Filters centrifuge tube, Sigma-Aldrich, New York, USA) and centrifuged at 14,000*g* for 10 min at 4° C. The supernatant was recovered and added to that previously collected. Afterwards, the supernatants were centrifuged at 14,000*g* for 10 min at 4° C, the supernatant was collected and 1.5× volume of 50 mM NH_4HCO_3 was added to the samples. This amount of 50 mM NH_4HCO_3 was based on the total volume of the samples, in order to reduce the concentration of urea and thiourea, because these may interfere with the action of trypsin. Subsequently, the samples were placed in Amicon tubes (Amicon Ultra-15 Centrifugal Filter Units, Merck Millipore Tallagreen, Ireland) and centrifuged at 5,000*g* at 25° C to a volume of approximately 150 μL.

The samples were then transferred to a new microcentrifuge tube, and a solution containing 5 mM DTT (Bio-Rad Laboratories, California, USA) was added for 40 min at 37 °C for reduction. Then samples were alkylated by the addition of 10 mM iodoacetamide (GE Healthcare, Upsalla, Sweden) for 30 min at room temperature in the dark. Digestion was then performed by incubation in 2% Trypsin (w/w) (Promega, Madison, WI, EUA) for 14 h at 37° C.

Tryptic digestion was stopped by the addition of 5% formic acid. Samples were de-salted and further purified using C18 spin column (Thermo Scientific, United States). An aliquot of each sample (1 μL) was removed for protein quantification by the Bradford method (Bio-Rad Bradford Assays, United States). The remaining was concentrated to approximately 2 μL in Speed Vac (Eppendorf, Hamburg, Germany) and ressuspended in 3% acetonitrile (ACN) with 0.1% formic acid, centrifuged at 14,000*g* for 10 min at 25° C and the supernatant was used for application in the nano Liquid Chromatagraphy Electron Spray Ionization Tandem Mass Spectrometric (nLC- ESI–MS/MS).

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