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Synergistic effects of proanthocyanidin, tri-calcium phosphate and fluoride on artificial root caries and dentine collagen

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ABSTRACT

Background: Proanthocyanidin has been shown to enhance dentine collagen stability and remineralization of artificial root caries.
Objectives: To evaluate the effect of proanthocyanidin (PA) in combination with tri-calcium phosphate (TCP) and fluoride (F) on resistance to collagen degradation and remineralization of artificial caries lesions.
Methods: Demineralized root fragments ($n = 75$) were randomly divided into five groups based on treatments: (i) 6.5% PA, (ii) TCP + F, (iii) TCP + F + 6.5% PA, (iv) 1000 ppm fluoride (Positive control) and (v) deionized water (control). Each specimen was subjected to pH cycling at 37° C for 8 days. Lesion depth and mineral loss were evaluated using microradiography and confocal laser scanning microscopy. The type of crystal formation was determined by XRD spectra. To evaluate the stability of root caries lesions against collagenase challenge, highly purified type VII collagenase from Clostridium was added to obtain a remineralizing solution that contained 7.5 U/mL collagenase and pH cycling was repeated. The different remineralizing solutions were collected after the pH cycling to assess the amount of hydroxyproline release. Collagen degradation depth and lesion depth were evaluated using transverse microradiography. Resistance to collagen degradation was determined using hydroxyproline assay. Data were analyzed using one-way ANOVA and Tukey multiple comparison tests.
Results: Results of one-way ANOVA showed that the test solutions had a significant effect on mineral loss ($p < 0.001$) and lesion depth ($p < 0.001$) of artificial root caries. The lowest lesion depth and mineral loss were observed in the TCP + F + PA ($p < 0.05$) group. The XRD patterns showed hydroxyapatite formation on TCP + F-treated artificial caries lesions, which were not altered by the addition of PA. The addition of PA to TCP + F significantly reduced collagen degradation depth, when compared to TCP only group ($p < 0.001$). Lesion depth was the lowest in the PA and TCP + F + PA groups following collagenase degradation ($p < 0.001$). The addition of PA to TCP + F also decreased hydroxyproline release, when compared to TCP + F group ($p < 0.001$).
Conclusion: The addition of PA to TCP + F reduced collagen degradation, inhibited demineralization and enhanced remineralization.

1. Introduction

According to the recent epidemiological survey, the majority of the world's population is ageing. With the improvement in general and oral health, the number of natural teeth retained in the elderly has increased over the years [1]. It has been reported that older adults experience root caries at a higher rate, similar to coronal caries in younger populations [2]. Moreover, with the higher prevalence of gingival recession in the elderly, the exposed enamel-cementum junction is at an increased risk of root caries [3]. Due to the rapid involvement of dentine in root caries and the inaccessibility of parts of root surfaces to restorative treatments, it is, therefore, necessary to find approaches to prevent or inhibit root caries progression [4–6].

Various approaches have been proposed for treatment of root caries [7]. Fluoride and fluoride-containing products have been shown to inhibit or lower the rate of root caries progression [7]. However, when root caries lesions extend to deep dentine, the application of remineralizing agents is more difficult and biofilm disruption is also much harder to achieve. The deep dentine lesions contain a matrix of organic components that are mostly collagen fibers with the loss of the inorganic component. The acidic challenge that occurs in carious lesion

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progression showed the presence of inferior mechanical properties in the root dentine compared with sound mineralized dentine [8,9].

When remineralization strategies are implemented to 'heal lesions', it is essential the collagen matrix is preserved. The collagen fibrils should also be strengthened to resist further breakdown in addition to providing the template for mineral deposition. However, clinically applicable intrafibrillar remineralization methods are still an experimental procedure [10]. Therefore, it is timely to find alternative ways to preserve the collagen matrix and remineralize carious lesions as a first phase of treatment. Unfortunately, remineralizing agents that are currently used, do not have a simultaneous collagen protective effect. For example, fluoride is a remarkable remineralization agent, but it has no intrinsic capability to inhibit collagen breakdown and strengthen the collagen matrix.

Various collagen cross-linkers have been evaluated to determine their preservation and strengthening of the dentine collagen matrix. Among them, proanthocyanidin (PA), a natural collagen cross-linker, has been shown to have multiple beneficial effects and has recently been proposed as a dentine bio-modifier [11–13]. As PA only has a weak remineralization capability, it is necessary to combine PA with a potent remineralization agents for cross-linking of collagen and remineralization of carious lesions [14].

Tri-calcium phosphate (TCP) is a precursor of hydroxyapatite [15]. Although TCP has been shown to remineralize enamel caries lesions, very few studies have quantitatively assessed the remineralizing effect of TCP on dentine caries [16,17]. Tri-calcium phosphate consists of a completely organic structure and it does not have any chemical interaction with PA. Within this study we have used a TCP containing paste together with 900 ppm fluoride. It is speculated that the combination of PA with TCP + F will maximize the remineralization effects of TCP and fluoride with the collagen cross-linking effect of PA. Hence, this study determined whether a synergistic effect of PA and TCP + F on remineralization of artificial root caries would occur. Furthermore, the cumulative collagen degradation resistance of PA and TCP + F was also studied. Thus, the null hypotheses were that the incorporation of PA with TCP + F had no effect on (i) lesion depth and mineral loss following the remineralization regimen and (ii) proteolytic biodegradation of collagen.

2. Materials and methods

2.1. Materials used

Tri-calcium phosphate used was from the 'anti-cavity' toothpaste (Clinpro™. Tooth Crème, 3 M ESPE). Proanthocyanidin was obtained from the International Laboratory of USA (>95% oligomeric proanthocyanidins) and fluoride as sodium fluoride (Sigma Aldrich, St. Louis, MO, USA). The demineralizing solution was prepared using 50 mmol/L acetic acid, 1.5 mmol/L $CaCl_2$ and 0.9 mmol/L KH_2PO_4 adjusted to pH 5.0 with KOH. The remineralizing solution consisted of 1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 130 mmol/L KCl and 20 mmol/L HEPES buffer and adjusted to pH 7.0 with KOH.

2.2. Preparation of dentine specimens

One hundred and twenty-five non-carious single rooted teeth were collected under the protocol approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster [HKU/HA HKW IRB] [UW11–242]. The crown and root apex were removed under water irrigation. One hundred and twenty five dentine blocks, approximately 5 mm × 5 mm × 5 mm in dimension, were prepared from the middle third of each root portion (Isomet, Buhler Ltd., Lake Bluff, IL, USA). The dentine surfaces were polished using ascending grits of silicon carbide papers (up to 2000) and the smear layer was removed by treating the dentine surface with 10% phosphoric acid solution for 10 s. Acid-resistant nail varnish was applied on the polished root surface to expose a window of 5 mm × 2 mm. One half of the window was covered to establish a baseline lesion. The dentine specimens were randomly distributed into two groups for pH cycling with ($n = 50$) and without collagenase stress ($n = 75$).

2.3. Methods of pH cycling

2.3.1. pH cycling without collagen stress

The prepared specimens ($n = 75$) were randomly divided into five groups ($n = 15$/group) based on the remineralization treatments: (i) deionized water (control), (ii) TCP + F, (iii) TCP + F + PA (iv) 6.5% PA and (v) 1000 ppm fluoride. The demineralizing and remineralizing solutions were prepared as stated above. All the solutions were freshly prepared. The test solution of 6.5% PA was prepared by adding PA to deionized water and the pH of the solution was adjusted to 7.0. A fluoride solution (1000 ppm) was prepared by dissolving NaF powder in deionized water. TCP was used as the commercially available form in the toothpaste obtained. In addition, PA (6.5% w/w) was added to TCP + F to prepare the TCP + F + PA group. Each sample was immersed in 37 °C demineralizing solution totaling 14 h, testing solutions or pastes for a total of 2 h and remineralizing solution totaling 8 h following the protocol used by Hiraishi et al. [18]. The samples were washed thoroughly with deionized water before immersion in each solution. The pH cycling was performed at 37 °C for 8 days, with six cycles per day. Upon completion of the pH cycling, the samples were thoroughly washed with deionized water prior to preparation for analysis.

2.3.1.1. Transverse microradiography. After 8 days of pH cycling, the mineral loss and lesion depth were determined using transverse microradiography. Samples ($n = 10$/group) were dehydrated using a series of ethanol solutions from 20% to 100%, followed by a transitional medium containing propylene oxide. Finally, the samples were embedded in epoxy resin and sectioned longitudinally through the lesion center into 200 ± 20 μm thick slices (Isomet, Buhler Ltd., Lake Bluff, IL, USA). The 200 ± 20 μm thick slices were X-rayed together with an aluminium step wedge at 12 kV and 4 mA for 45 s. Microradiographic plates were processed (Eastman Kodak Co., Rochester, N.Y., USA) and the radiographic images were taken from the microscope to the computer with a CCD camera. Lesion depth was defined as the distance from the surface to the lesion where the mineral content was >95% of the sound dentine. Mineral loss was determined by plotting the vol% mineral profile towards lesion depth in each sample with the sound dentine set at 48 vol% mineral content [19]. One-way ANOVA and Tukey multiple comparisons were used to analyze the effect of various remineralizing treatments on lesion depth and mineral loss ($\alpha = 0.05$).

2.3.1.2. Confocal laser scanning microscopy. The remaining samples ($n = 5$/group) without dehydration were cut and embedded in epoxy resin. The samples were then further sectioned into 200 ± 20 μm thick slices using a diamond wafering blade under water cooling (Isomet, Buhler Ltd., Lake Bluff, IL, USA). The prepared dentine slices were stained with 0.1% Rhodamine B solution (Aldrich Chem. Co., Milwaukee, WI, USA) for 1 h, and rinsed three times with deionized water then analyzed with a CLSM (Zeiss LSM 510, Carl Zeiss, Inc., Germany), using an argon laser with a 529 nm excitation wavelength. Areas were scanned 30 μm below the cut surface to reduce the influence of the smear layer created during the cutting and polishing procedures.

2.3.1.3. Crystal characterization. A Bruker D8 Advance X-ray powder diffractometer with CuKa (l = 1.5418 A° radiation equipped with a scintillation counter was used to collect step-scanned lock-coupled X-ray diffraction (XRD) data from two specimens from each group. An accelerating voltage of 40 kV was used with the applied current of the X-ray generator limited to 40 mA. A Göbel Mirror was used to parallel the X-ray beams and confined by a divergence-limiting slit (0.6 mm). Then a Soller slit was used to reduce the axial divergence of the incident