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Research paper

Effect of proanthocyanidin on ultrastructure and mineralization of dentine collagen

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ABSTRACT

Objective: Proanthocyanidin (PA) is a natural collagen cross-linker that has been used in dentine matrix biomodification for reparative and preventive therapies. This study evaluated the ultrastructure of collagen after its interaction with PA. Furthermore, the mineralization of PA-biomodified collagen matrix was observed.

Methods: Ten freshly extracted sound human molars were sectioned into 0.5 mm × 1.7 mm × 7 mm beams for ultrastructural evaluation of PA and dentine matrix under Field Emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM). Specimens for TEM were completely demineralized and divided into three groups according to PA treatments: deionized water, 2% PA and 6.5% PA. The specimens were fixed, dehydrated, sectioned and examined using TEM. Specimens for FESEM were lightly conditioned with EDTA and similarly divided into the three groups for observation using FESEM. Type I collagen from calf skin was used to analyse the mineral interaction after treatment with 6.5% PA. Formvar- and carbon-coated 400-mesh Ni grids (EMS, Hatfiels, PA, USA) were placed over a 2 mg/mL collagen solution prepared from calf skin-derived Type I collagen to achieve self-assembly of collagen fibrils. Grids were treated with 6.5% PA and divided into two groups. One group was floated over a remineralization solution containing 20 mM HEPES, 2.25 mM $CaCl_2$-$2H_2O$, 1.35 mM KH_2PO4, 3.08 mM NaN3 and 130 mM KCl and the other group was over a CPP-ACP solution (Tooth mousse 1:100 dilution with deionized water). The floating samples were kept in a 37 °C and 100% humidity chamber. Grids were taken out at selected time durations (24 h, 48 h and 72 h for mineralization solution/24 h for CPP-ACP) and observed under TEM without staining. Selected area electron diffractions (SAEDs) were performed at 110 kV.

Results: Following treatment of demineralized dentine collagen matrix with PA, the size and number of interfibrillar spaces were reduced. The collagen fibrils aggregated together with a reduction in porosity. A characteristic banding pattern of collagen fibrils was observed under TEM. Treatment of PA-biomodified collagen fibrils with remineralization solution increased mineral aggregation along its long axis, when compared to the control group. Furthermore, treatment of PA-biomodified collagen fibrils with CPP-ACP solution enhanced mineral uptake and deposition as well as initiated apatite formation within 24 h.

Conclusion: Proanthocyanidin alters the ultrastructure of demineralized dentine collagen matrix. The PA-biomodified collagen matrix promotes remineralization.

1. Introduction

Type I collagen is the most abundant type of collagen found in the body. It is a fibrillar collagen, which is strong, elastic and arranged in a highly organized hierarchical structure (Antoine, Vlachos, & Rylander, 2014; Gelse, Poschl, & Aigner, 2003). The dentine matrix mostly consists of Type I collagen, which provides the tissues with strength and form (Kinney, Marshall, & Marshall, 2003). The collagen fibrils, approximately 30% by volume, are roughly 50–100 nm in diameter and randomly oriented in a plane perpendicular to the direction of dentine formation (Goldberg, Kulkarni, Young, & Boskey, 2011). The minerals occupy two sites within this collagen scaffold: intrafibrillar (inside the periodically spaced gaps in the collagen fibril) and extrafibrillar (in the interstices between the fibrils). The partition between these two sites is unclear, although it is believed that between 70 and 75% of the minerals may be extrafibrillar (Bonar, Lees, & Mook, 1985; Pidaparti,

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Chandran, Takano, & Turner, 1996). The mineral crystallites are needle-like near the pulp with the shape progressing to plate-like within the proximity to enamel (Kinney, Pople, Marshall, & Marshall, 2001). The thickness of the crystallites is about 5 nm and varies with location.

Dentine matrix can be destroyed or damaged in various ways, such as trauma, erosion or dental caries. Since there is a limited ability for these tissues to remodel, the current treatment option is to restore it using synthetic biomaterials. However, the reparative procedure faces numerous challenges due to the presence of a weakened collagen matrix. Recently, biomodification of dentine using collagen cross-linkers has been introduced to enhance the mechanical stability of dentine. Various synthetic agents, natural materials as well as physical methods have been identified for having a cross-linking effect on collagen.

Among the natural collagen cross-linking agent, proanthocyanidin (PA) is a promising agent for maintaining collagen integrity. Proanthocyanidin belongs to a category referred to condensed tannins that are highly hydroxylated structures, capable of forming an insoluble complex with carbohydrates and proteins (Cao, Fu, & He, 2007). The chemical interaction of PA and collagen is believed to result from the formation of hydrogen bonds between the protein amide carbonyl and the phenolic hydroxyl groups (Hagerman & Klucher, 1986). In addition, covalent and hydrophobic bonds are also involved in the interaction.

Biomodification of demineralized dentine matrix using PA has enhanced the mechanical properties of dentine matrix, strengthened the hybrid layer and increased resin-dentine bond strength (Bedran-Russo, Pereira, Duarte, Drummond, & Yamauchi, 2007; Castellan, Pereira, Grande, & Bedran-Russo, 2010; Castellan, Bedran-Russo, Karol, & Pereira, 2011). Proanthocyanidin increased bond durability under thermal cycling as well as enzymatic challenge (Broyles, Pavan, & Bedran-Russo, 2013; Hass, Luque-Martinez, Gutierrez et al., 2016; Hass, Luque-Martinez, Munoz et al., 2016; Liu et al., 2014). Furthermore, several recent reports have proven the ability of PA to reduce caries progression by inhibiting biofilm formation and organic acid production (Zhao et al., 2014). Although PA preserved the collagen scaffold and enhanced remineralization of artificial root caries, its remineralization effect is lower than fluoride (Xie, Bedran-Russo, & Wu, 2008). Hence, more recently, PA has been used as a remineralization promoter (Epasinghe, Yiu, & Burrow, 2015). Together with the remineralizing agents, PA enhanced mineral deposition in artificial caries lesions (Epasinghe, Yiu, Burrow et al., 2015). However, no study has reported the effect of PA on the ultrastructure and mineralization of Type I collagen. Thus, this study aimed to qualitatively observe the ultrastructural features of PA-biomodified Type I collagen and its interaction with minerals under field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM).

2. Methods

2.1. TEM observation of cross-linking of dentine collagen matrix

Five freshly extracted caries-free human molars were collected after the patients' informed consent was obtained under a protocol reviewed and approved by the Institutional Review Board, the University of Hong Kong (UW 11-242). Teeth were disinfected with 0.5% chloramine T, cleaned and occlusal enamel was removed using a slow speed saw under water irrigation (Isomet, Buhler Ltd., Lake Bluff, IL, USA). The root portions of the teeth were sectioned 1 mm beneath the cemento-enamel junction. The teeth were sectioned into 0.5 ± 0.1 mm thick slices using a slow speed diamond wafering blade under water cooling and then trimmed into a rectangular shape with the dimensions of 0.5 mm x 1.7 mm x 7 mm using a high speed handpiece. Specimens were immersed in 10% phosphoric acid solution (LabChem Inc., Pittsburgh, PA, USA) for a period of 5 h and thoroughly rinsed with distilled water for 10 mins (Bedran-Russo, Castellan, Shinohara, Hassan, & Antunes, 2011). X-rays were taken of the specimens to verify complete tissue demineralization. They were then divided into three groups (n = 5) and treated with following solutions in room temperature for 30 mins.

1. Deionized water
2. 2% PA
3. 6.5% PA

PA was obtained from the International Laboratory of USA (> 95% oligomeric proanthocyanidins). The testing solutions of 2% and 6.5% PA were prepared by adding PA to deionized water and the pH of the solution was adjusted to 7.0. All specimens were rinsed with distilled water for 30 s. Specimens were fixed in 2.5% paraformaldehyde/glutaraldehyde in 0.1 M sodium cacodylate for 3 days, then rinsed and dehydrated in increasing concentrations of ethanol (50%–20 min, 75%–20 min, 90%–30 min, 95% twice − 30 min, 100% twice − 60 min). Specimens were infiltrated with LR White resin (Electron microscopy Sciences, Hatfield, PA). Specimens were sectioned into 70 nm thick sections using a diamond knife attached to an ultra-microtome (Ultracut UCT, Leica, Buffalo Grove, IL). Sections were placed on copper grids and post-stained with uranyl acetate for 1 min. Sections were imaged and analysed on TEM (Philips CM100, Tokyo, Japan) at 80 kV.

2.2. FESEM observation of cross-linking of dentine collagen matrix

Fifteen longitudinal sections with the dimensions of 0.5 mm × 1.7 mm × 7.0 mm were obtained from five recently extracted sound human teeth as mentioned previously. The dentine specimens were then ground with 600-grit wet abrasive paper for 60 s to reduce the thickness and to produce a standard smear layer on the surface. The dentine was then treated with EDTA (0.5 M, pH 7.0) for 30 mins, followed by 10% citric acid for 30 mins, then rinsed using a saline solution for 5 s. The specimens were similarly divided into three groups (n = 5) and treated with following solutions in room temperature for 30 mins:

1. Deionized water
2. 2% PA
3. 6.5% PA

All specimens were rinsed with distilled water for 30 s, fixed with 2.5% glutaraldehyde and dehydrated using ascending ethanol series (5%–100%). The samples were critical point dried with hexamethyldisilazane for 30 mins followed by 1 h in a vacuum chamber. Finally, the specimens were coated with carbon and the surface was examined under a FESEM (Hitachi S-4800 FEG, Tokyo, Japan).

2.3. Mineralization of Type I collagen

PA is commonly used at 6.5% for its collagen cross-linking effect and is therefore chosen for mineralization assessment. Formvar- and carbon-coated 400-mesh Ni grids (EMS, Hatfiels, PA, USA) were placed over a 2 mg/mL collagen solution prepared from calf skin-derived Type I collagen (Sigma-Aldrich). Self-assembly of collagen fibrils was achieved by neutralizing the collagen solution with ammonia vapour (7% v/v NH_4OH for 4 h). The grids were divided into groups and treated with different concentrations of PA (0.5%, 2%, 6.5% and 10%) for 30 min. The fibrillar assembly was observed under TEM (Philips CM100, Tokyo, Japan) at 110 kV after staining with uranyl acetate. Unstained grids containing PA-treated collagen fibrils were divided into two groups, one group was floated over a mineralization solution containing 20 mM HEPES, 2.25 mM $CaCl_2$-$2H_2O$, 1.35 mM KH_2PO_4, 3.08 mM NaN_3 and 130 mM KCl and the other group was over a CPP-ACP solution [(Tooth Mousse, GC Japan), 1:100 dilution with deionized water]. The floating samples were kept in a 37 °C and 100% humidity chamber. Grids were taken out at the selected time duration (24 h, 48 h