Bio-Modification of Demineralized Dentin Collagen by Proanthocyanidins-enriched Cranberry: An In-Vitro Study

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Abstract

The aims of this study were to evaluate the effect of cranberry juice (CB) on demineralized dentin collagen by evaluating the mechanical properties of the collagen and compare its efficacy to glutaraldehyde (GA).

The effect of 100%, 50%, 25% pure CB, and 0.5% GA were tested in this experiment. The water absorption of collagen was evaluated by swelling ratio (SR) test of (0.5mm x 1.5mm x 6mm) size demineralized dentin beam treated with an experimental solution. The strength of collagen was evaluated by the ultimate tensile test (UTS) of (0.5mm x 1.5mm x 6mm with a neck size of 0.5mm) size demineralized dentin beam treated with an experimental solution. The demineralized dentin block with an approximately (1.5 x 2.5 mm) window size was tested for an 18hour collagenase challenge by light microscopy.

One-way ANOVA showed a significant effect of the tested solution on SR, UTS, and collagen degradation ($p < 0.001$). The 100% and 50% CB showed statistically significant effects on water sorption, ultimate tensile strength, and amount of collagen degradation compared to the control group ($p<0.05$)

The cranberry juice has the potential to improve the mechanical properties of collagen and prevent degradation of demineralized dentin collagen against enzymatic degradation.

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Introduction

Dentin is the main bulk of the tooth composed of approximately 70% mineral, 20% organic component and 10% fluid by weight. Type I collagen fiber accounts for 90% of the organic matrix[1] . In physiological conditions, dentinal collagen fibers are surrounded by hydroxyapatite. However the demineralization process of dentin exposes the collagen fibers which are prone to degradation by enzyme[2]. In the early stages of dentin caries, minerals are dissolved by a fine gradient from the outer surface, while the characteristic cross-banding of the collagen fibers is maintained[3]. The demineralized dentin collagen serves as a scaffold for colonizing cariogenic bacteria. At more advanced stages of degradation, the exposed collagen is broken down by proteolytic enzymes, and collagen fibers lose their structural integrity[4]. However, a recent study by Tjäderhane et al., 2015 suggested that collagen may lose the cross-banding at early stage of demineralization, in which an exposed telopeptide region of the collagen molecule can be degraded by host-derived collagenolytic enzymes [5].

The bio-modification of demineralized dentin is an essential step to arrest the progression and promote re-mineralization of caries lesions. Preservation of dentinal collagen from enzymatic degradation, maintenance of hydration channel around collagen and strengthening of the collagen might initiate a suitable condition for re-mineralization of the demineralized dentin. Caries prevention by food or drink is an easy and effective approach to achieve an impact on mass population.

Cranberry is a native North American fruit poses various beneficial properties for human health, such as the inhibition of human cancer

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cell line proliferation and the prevention of adherence of urinary tract infectious agents[6–8]. Cranberry is a good source of Proanthocyanidins (PA) easily available in nature.[9] In the area of dental research, it has been reported that a high molecular- weight cranberry fraction inhibits the aggregation of many oral bacteria and affects dental biofilm formation[10,11]. Moreover, it reduces streptococcus mutans levels in saliva, inhibits in vitro adhesion of streptococcus sobrinus to hydroxyapatite, and promotes streptococcus sobrinus desorption on artificial biofilms[12–14]. Discovery of these health benefits made it a popular drink; however, the caries prevention or effect on demineralized dentin has not been sufficiently evaluated.

The aim of this study was to assess the potential role of cranberry juice on modification of demineralized dentin by studying its effect on the degradation of demineralized dentin collagen by collagenous enzyme, maintenance of hydration channel around demineralized collagen and strengthening of demineralized collagen. The null hypotheses tested were (I) cranberry juice has no effect on water sorption of demineralized collagen, II) cranberry juice on strengthening of demineralized collagen and (III) cranberry juice has no effect on preservation of demineralized dentin collagen.

Materials and methods

Preparation of dentin specimen and tested chemicals

100% pure cranberry juice without sugar and additive (Junjo-sen Cranberry 100 NFC INR60905) were purchased from Marukai Corporation, Japan. The glutaraldehyde solution (wako pure chemical company) was used as the control in all conducted experiments. The test solutions used in this study were 100% CB, 50% CB, 25% CB, 0.5% GA and DW as a control. The CB juice was diluted with deionized water. Thirty-six freshly extracted intact human molars were used in this study. Teeth were trimmed with model trimmer to remove enamel. The teeth were then sectioned longitudinally into serial slab which were further sectioned to obtain (0.5mm x 1.5mm x 6mm) dentin beams by means of a low-speed diamond saw Isomet (Buehler Ltd., Lake Bluff, IL, USA) with water cooling. Thirty beams were used for swelling ratio test (n=6/group). The remaining 30 beams were further trimmed in the middle with dental bar to obtain hourglass shape specimen with a neck diameter of 0.5mm x0.5mm for UTS. For collagenous challenge experiment, dentin blocks (4 mm X 4 mm) were obtained by sectioning the root of the molar teeth by means of a low-speed diamond saw Isomet under water cooling. The dentin blocks were then polished with 1000# grit paper to remove the cementum and create a flat surface, followed by embeddeding the blocks in self-curing acrylic resin (shade A2, UNIFAST II; GC, Tokyo, Japan) and the dentin surfaces were polished using 2000# grit papers (Sankyo, Saitama, Japan). The polished tooth surfaces were covered with acid-resistant nail varnish leaving an uncovered window approximately 1.5 x 2.5 mm in diameter. The areas covered with nail varnish served as the baseline for sound dentin. Then, to create an incipient lesion the specimens were demineralized with acetate buffer (0.1 mol/L, ph 4.3) for 3 days at 37°C (100 ml/10 specimens).

Swelling ratio testing

The specimens used for this test were immersed in10% phosphoric acid solution for 5 h at room temperature to complete demineralization and thoroughly rinsed with distilled water for 10 min. The demineralized specimens (n = 6) were treated for 12 h at 370C either in the test solutions, GA or distilled water. After treatment, the specimens were kept in water and equilibrated overnight in phosphate buffered saline (pH 7.4) at room temperature. The specimens were blotted with filter paper to remove excess surface water after removal and weighed immediately. Then the dentin specimens were placed in distilled water for10 min to remove the buffer salts. They were dried in a desiccator to a constant weight and weighted. The swelling ratio was calculated as the ratio of the weight of swollen specimen to that of dry specimen.

Ultimate tensile strength (UTS) testing

The hourglass-shaped specimens obtained from human molar teeth were immersed in 10% phosphoric acid solution for 5 h at room temperature to complete demineralization and thoroughly rinsed with distilled water for 10 min. The specimens (n = 6) were treated in each test solution, GA or distilled water at 370 C for 12 h and were subjected to UTS evaluation. The specimens were glued to a jig using an adhesive

(MODEL REPAIR II BLUE, DENSPLY-Sankin, Ohtawara, Japan) and stressed to failure under tension using a universal testing machine (EZ Test, Shimadzu Co., Kyoto, Japan) at a crosshead speed of 1 mm/min.

Collagenase challenge an assessment of degraded depth by light microscopy

The collagenase enzyme (type IA, Clostridium histolyticum C-9891, Sigma-Aldrich, Saint Louis, MO, USA) solution contained 50 mmol/L PIPES, 150 mmol/L Nacl and 5 mmol/L Cacl2 adjusted to pH 6.5. The demineralized specimens (n = 8) were treated for 5 min either in the test solutions, GA or distilled water. The specimens were then gently washed with phosphate buffer solution. Each specimen was placed in 24 wells cell culture plate containing 1.5 ml (1 U/ml) collagenase solutions for 18h. After collagenase challenge, the specimens were washed for 5 min and sectioned by a low-speed diamond saw into thin sections 200-220 µm and gently polished using 2000# papers. Then the sections were placed in between a glass slide and cover slide by adding a droplet of deionized water. The images of the specimens were captured by the camera connected to the polarized microscope (SMZ1000, Nikon Corp., Tokyo, Japan). The total area of the degraded collagen and the width of the window were measured by image processing software (Image J 1.42). The mean depth of degraded collagen matrix was estimated by dividing the total area of the degraded collagen by its window width[15].

Statistical analysis

The effects of test solution on SR, UTS and collagen degradation data were tested by one-way ANOVA using a statistical software package (Sigma Stat Version 16.0, SPSS, Chicago, IL, USA). In case of significant, further statistical analyses were performed by Tukey multiple comparisons test and the level of statistical significance was set at 5%.

Results

One-way ANOVA showed significant effect of tested solution on SR, UTS and collagen degradation ($p < 0.001$).

Figure 1 presents the results of the SR. Tukey post hoc test revealed that the SR of 100% CB treated group and 50% CB treated group were significantly lower than the control group. The 25% CB treated group and 0.5% GA showed a statistically insignificant SR with control and other test groups.

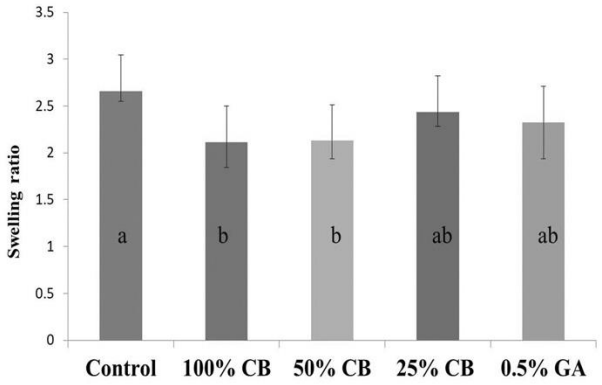

Figure 1. Swelling ratio.
Groups identified with same letters are not statistically significantly different (p>0.05).

The mean UTS values for demineralized dentin beams are shown in Figure 2. The UTS of 100% CB treated group and 50% CB treated group were significantly higher than control and the other tested groups. The 25% CB treated group and 0.5% GA showed statistically insignificant UTS when compared to that of the control group.

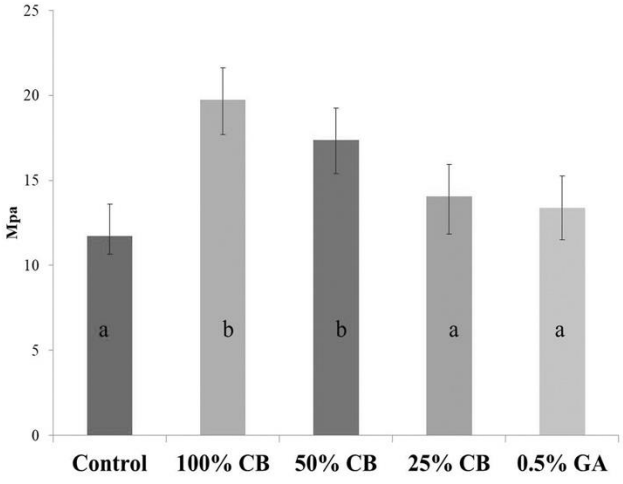

Figure 2. Ultimate tensile strength in MPa.
Groups identified with same letters are not statistically significantly different (p>0.05).

The LM analysis revealed that the collagen degradation was significantly reduced by the application in tested solutions when compared with control group (Figure 3 and

Figure 4). The 100% CB treated group and 0.5% GA treated group showed significantly lower depth of collagen degradation when compared with 25% CB treated group however the depth of degradation was statistically insignificant compared to 50% CB treated group ($p < 0.001$).

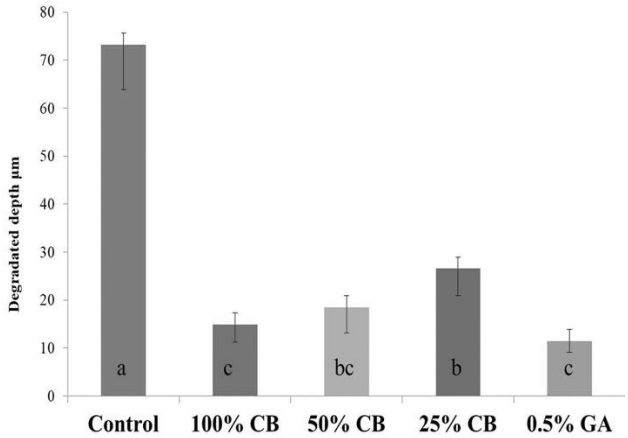

Figure 3. Depth of collagen degradation in µm. Groups identified with same letters are not statistically significantly different (p>0.05).

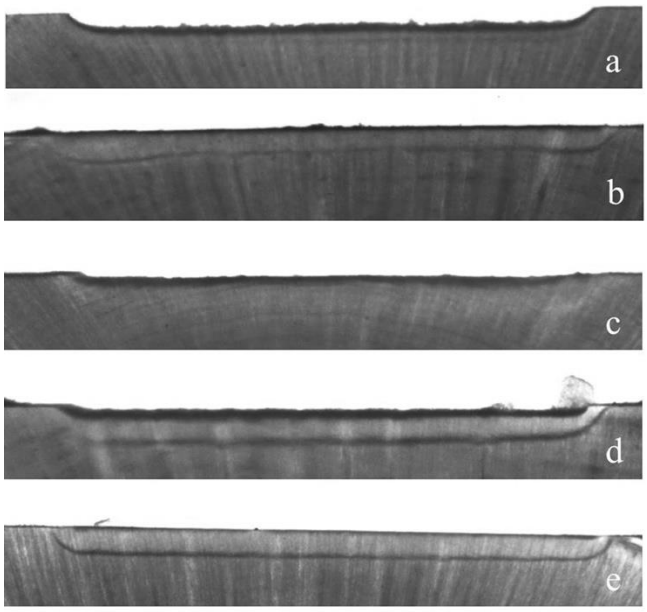

Figure 4. Reprehensive light microscopy images of dentin section: a) control; b) 100% CB; c) 50% CB; d) 25% CB; e) 0.5% GA.

Discussion

The results of this study showed that certain concentration of CB juice showed a significant effect on swelling ratio and UTS of dentin beams, thus the first and the second null hypotheses were partially rejected. All use concentrations of CB juice had a significant effect in preservation of demineralized dentin collagen, thus the third hypothesis was rejected.

In our study the control group treated with water showed a greater degree of water sorption when compared to the cranberry groups. Previous study demonstrate that the water molecule in dentin collagen is either tightly bound to specific sites on collagen chains or it fills the spaces between the collagen molecules[16]. The inter-molecular and inter-chain bonds are formed by inherent water molecules that forms multi span hydrogen bonded bridges by connecting the neighboring collagen molecules[17]. According to the fact, there is a general agreement that triple-helices structures are surrounded by a highly structured “cylinder of hydration’’. The lateral separation in macromolecular assemblies of fibriler units in type I collagen regulate by the diameter of these “cylinders’’. Acid attacks either from bacteria or during etching for adhesive restoration create porosity and nano-voids at the fibril surface, where water molecules can be sequestered, leading to solvent uptake and elution[18]. The cranberry fruit is a rich source phenolic compound like proanthocyanidins (PA)[19]. In drug–protein interaction theory, there are four types of non-covalent interactions between ligand and protein, i.e. hydrophobic effect, hydro-gen bond, van der Waals force and electrostatic interaction[20]. The PA molecule present in cranberry bound to the demineralized collagen fiber may create a zone of hydrophobicity around it which prevents the solvent uptake and elution on via porous fibril surface.

The ultimate tensile strength of collagen represents the crosslinking and structural integrity of collagen fiber[21]. The water sorption of demineralized collagen increases the diameter of “hydration cylinders’’ and lateral separation in the macromolecular assemblies in fibriler units of type I collagen leading to breakdown of the native cross-linking of collagen molecule. The low UTS value obtained in control group might be the consequence of the above fact[22]. On the other hand cross-linking molecule like PA has been demonstrated to reveal their interaction with proteins[23]. PA contains a phenyl ring bearing a hydroxy group and possesses a phenol function that constitutes an amphiphilic moiety. The amphiphilic property of these polyphenols shows combination of hydrophobic character of its

planar aromatic nucleus, with the hydrophilic character of its polar hydroxy substituent. The hydrophobic portion in aromatic rings induces a p-stacking (van derWaals) interaction with the hydrophobic counterpart in other molecules; while the hydrophilic portion is polarized and capable of hydrogen bonding[24] This hydrogen bond in cranberry treated groups might contribute to increase the ultimate tensile strength of demineralized dentin collagen.

The proteolytic degradation of collagen takes place due to de-fibrillation and cleavage of collagen by proteolytic enzyme[25]. PA has the capability to prevent proteolytic degradation. This might be achieved by neutralizing the enzyme of by competing with proteolytic enzyme to bind with site of cleavage[26]. Previous studied showed similar effects of other collagen cross-linkers to prevent proteolytic degradation of dentin collagen[27].

Conclusions

Within the limitation of in vitro study it can be conclude that, pure cranberry juice has the potential of bi-modification of demineralized dentin collagen. This potential benefit of cranberry juice might be useful for caries prevention. However, in vivo studies are warranted to confirm the alleged beneficial effect of this juice on tooth structure.

Declaration of Interest

The authors report no conflict of interest.

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