Effects of Natural Cross-Linkers on the Stability of Dentin Collagen and the Inhibition of Root Caries in vitro

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Collagen degradation · Cross-linkers · Genipin · Glutaraldehyde · Proanthocyanidin · Root caries

Abstract
Purpose: To evaluate the effects of dentin collagen modifications induced by various cross-linkers on the stability of collagen matrix and the inhibition of root caries. Materials and Methods: The following cross-linkers were tested: 5% glutaraldehyde (GA), 0.5% proanthocyanidin (PA), 0.625% genipin (GE). In the first experiment, cross-linker-treated demineralized human root dentin was digested with bacterial collagenase, centrifuged, and the supernatants were subjected to amino acid analysis to determine collagen content. The residues were analyzed by SDS-PAGE and hydroxyproline analysis. In the second experiment, bovine root surfaces were conditioned with phosphoric acid, treated with the cross-linkers, incubated with Streptococcus mutans and Lactobacillus acidophilus for 1 week and the root caries inhibition was evaluated with confocal microscopy. Lastly, the ability of the bacteria to colonize the root surface was evaluated. In this experiment slabs of bovine root were treated with the cross-linkers and incubated in a suspension of S. mutans and L. acidophilus. The slabs were washed, resuspended in water, glucose was added, and the pH measured.

Results: While all collagen was digested with collagenase in the control groups, only a small proportion was solubilized in the GA-, PA-, and GE-treated groups. The root caries was significantly inhibited by treatment with PA or GA. Drops in pH in the cross-linker-treated groups were essentially the same as in the untreated group. Conclusion: Naturally occurring cross-linkers, especially PA, could be used to modify root dentin collagen to efficiently stabilize collagen and to increase its resistance against caries.

Preventive methods such as fluoridation of public water have helped control caries prevalence over the last decades [McDonagh et al., 2000]. However, an increasing elderly population has raised the issue of an increase in root caries in dentistry. With aging gingival recession often exposes the root surface, which is more susceptible to caries than enamel. According to a recent meta-analysis, the elderly population shows high rates of root caries [Griffin et al., 2004]. Some studies have been performed to reduce collagenase activity by pharmacological means with the objective of preventing caries [Sulkala et al., 2001; Tjaderhane et al., 1999]. Here, with the same objective, we have taken a different approach, i.e. modifying dentin collagen matrix to...
improve its stability in the hope that this may help prevent matrix degradation. Fixation of biological tissues, which stabilizes the organic matrix, has been extensively studied in the past. Various cross-linking reagents such as glutaraldehyde (GA), formaldehyde, carbodiimide, and epoxy compounds, have been used for tissue fixation; however, because of toxicity and/or instability with time, their use in vivo has been limited [Cheung et al., 1985; Nimni et al., 1988]. Recently, naturally occurring cross-linkers including proanthocyanidins (PA) and genipin (GE) have been proven to be biocompatible and stable for a long period of time in animals [Han et al., 2003; Sung et al., 1998]. PA is a naturally occurring plant metabolite widely available in fruits, bark, leaves and seeds. The PA used in this study originated from grape seeds. They are also known as condensed tannins and their structure varies depending upon the nature of the flavan-3-ol building blocks of PAs [Dixon et al., 2005]. GE is derived from gardenia fruits. Those compounds, as cross-linkers of connective tissues such as tendon and pericardium, are as efficient as GA but possess significantly less toxicity [Han et al., 2003; Sung et al., 1998, 1999, 2001; Tsai et al., 2000].

In this study, we investigated the potential effects of PA and GE on the stabilization of dentin collagen and the inhibition of root caries. The effects were compared to those of GA and control groups. The null hypothesis tested was that GE and PA would not prevent solubilization of collagen and root caries as well as GA.

Materials and Methods

Collagen Stability

Collagen stability was assessed by the digestibility by bacterial collagenase of dentin collagen, with or without treatment with cross-linkers. Twelve freshly extracted intact human third molars were used for this study. The roots were separated from the crowns using a low-speed diamond saw (Isomet, Buehler, Ltd., Lake Bluff, Ill., USA) under water cooling. The roots were further sliced 3 mm below the cementoenamel junction (CEJ) and their upper portions were used for the analysis. The cementum and pulp were removed using high-speed diamond burs under water cooling. The root dentin was pulverized in liquid N2 by a Spex Freezer Mill (SPEX CertiPrep, Inc., Metuchen, N.J., USA), washed with distilled water, and lyophilized. In order to obtain sufficient quantities of collagen for biochemical and statistical analyses, the root dentin was pulverized in liquid N2 by a Spex Freezer Mill (SPEX CertiPrep, Inc., Metuchen, N.J., USA), washed with distilled water, and lyophilized. In order to obtain sufficient quantities of collagen for biochemical and statistical analyses, three teeth were combined as a group and four groups were generated. After pulverization, the samples were demineralized with 0.5 M EDTA (pH 7.4) for 10 days at 4 °C, extensively washed with distilled water and lyophilized. Ten 2-mg aliquots of demineralized dentin matrix were made in each group (i.e. a total of 40 aliquots in four groups) and allocated to the following five treatment groups: untreated controls; controls treated with phosphate-buffered saline (PBS); 5% GA; 0.5% PA, and 0.625% GE.

GA (Fisher Scientific, Pittsburgh, Pa., USA), PA (Polyphenolics, Madera, Calif., USA), and GE (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were dissolved in PBS. A 2-mg aliquot of demineralized dentin matrix was added to 1 ml of each cross-linker solution and incubated for 72 h at 4 °C for GA and PA, and at 37 °C for GE. These conditions, i.e. concentrations, temperatures and time of incubation, have been reported to be optimal for cross-linking induction [Han et al., 2003; Liang et al., 2004]. As controls, aliquots were left untreated for 72 h at 4 °C or incubated in PBS.

After treatment, the samples were extensively washed with distilled water, suspended in 0.5 ml of ammonium bicarbonate with distilled water, and lyophilized. In order to obtain sufficient quantities of collagen for biochemical and statistical analyses, the root dentin was pulverized in liquid N2 by a Spex Freezer Mill (SPEX CertiPrep, Inc., Metuchen, N.J., USA) for 15 s and thoroughly rinsed with distilled water by shaking at 37 °C in the supernatants and residues in each group.

For the supernatants, all eight samples were hydrolyzed with 6 N HCl and subjected to amino acid analysis to determine the hydroxyproline content using a Varian/Waters HPLC system (Varian 9050 and 9012; Varian, Inc., Walnut Creek, Calif., USA) fitted with a strong cation exchange column (AA911; Transgenic, Inc., San Jose, Calif., USA) [Yamauchi and Shiiba, 2002]. The collagen content (μg) was calculated based on the values of 300 residues of hydroxyproline in a collagen molecule, and a molecular weight of collagen of 285 kDa. Means and SD were calculated and data were analyzed by one-way ANOVA and Fisher’s PLSD using Statview 5.0.1 software (SAS Institute, Inc., Cary, N.C., USA) with a significant level of 5%.

For the residues, 6 samples in each group were characterized by SDS-PAGE and by hydroxyproline analysis. For the former, aliquots were dissolved in SDS sample buffer, boiled for 2 min, centrifuged, and the supernatants were subjected to SDS-PAGE with a 6% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie brilliant blue, visualized and photographed under a light box. Another two sets of residues were hydrolyzed with 6 N HCl and subjected to hydroxyproline analysis as previously described.

Root Caries Inhibition

Fifteen lower bovine incisors were used in this study. Roots were separated from the crowns at the CEJ using a low-speed diamond saw (Isomet) under running water. Pulpal tissue was removed and the roots cleaned of soft tissues. The mesial and distal surfaces of the roots were ground mechanically (Ecomet 3, Buehler, Ltd.) under running water with 600-grit silicon carbide paper to obtain a flat dentin surface. Nail varnish was applied to the entire root surface except for two windows, ~10 mm × 2 mm, on the flattened mesial and distal surfaces. Exposed root surfaces were conditioned with 37% phosphoric acid (Scotchbond Etchant, 3M ESPE, St. Paul, Minn., USA) for 15 s and thoroughly rinsed with tap water. Of 15 roots, 3 were set aside (‘phosphoric acid’ group) and 12 randomly assigned to one of the following four groups (3 roots/group): 5% GA, 0.5% PA, 0.625% GE, or ‘no treatment’. The ‘phosphoric acid’ roots were not incubated in the bacterial solution but kept in distilled water by shaking at 37 °C in the dark, and used to evaluate the depth of demineralization by the acid only.

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The ‘cross-linker’ and ‘no treatment’ groups were immersed in PBS with and without cross-linkers, respectively, incubated for 72 h, thoroughly washed under running tap water and incubated in a bacterial suspension containing Streptococcus mutans serotype c (ATCC 10449) and Lactobacillus acidophilus (ATCC 4356). A stock culture of each bacterium was transferred to trypticase soy broth (TSB, Difco Laboratories, Inc., Tucker, Ga., USA) and incubated at 37°C in ambient atmosphere. After 48 h, this suspension was transferred to a fresh TSB tube, the bacteria mixed in equal amounts and the suspension adjusted with fresh TSB to A(660) = 0.1. The suspension was then supplemented with sucrose to a final concentration of 2.5% and the prepared root specimens immersed in 200 µl of this solution. The specimens were incubated at 37°C for 1 week and the sucrose-supplemented medium was replenished every 48 h.

Following incubation, specimens were sectioned perpendicular to the long axis of the roots using a low-speed diamond saw (Isomet) under running water. Three slices approximately 0.35 mm thick were obtained from each root, the first one being approximately 2 mm below the CEJ. Since there were windows at both sides of the roots, a total of six views of the carious lesions were obtained from each tooth, generating 18 views/group (i.e., 3 roots/group). Some of the sections that partially contained CEJ were eliminated, thus, 14–17 views/group were evaluated. Specimens were left overnight at room temperature to dry and then stained for 24 h with rhodamine B (Allied Chemical, Morristown, N.J., USA), buffered with KOH, at pH 7.0.

Confocal laser microscopy (Zeiss LSM5 Pascal CLSM, Carl Zeiss, Inc., Thornwood, N.Y., USA) was used to analyze the stained surfaces and to determine the extent of the lesions. In this study a Zeiss LSM5 Pascal CLSM with a ×5, 0.15 NA, Plan-Neofluor lens was used. Excitation was with the 543-nm line of a green HeNe laser. The pinhole setting was at 1 airy unit, scanning a 512 × 512 pixel raster at a zoom setting of 1. Image analysis was performed using ImageJ 1.36b (National Institutes of Health, Bethesda, Md., USA). An imaginary line connecting the edges of the lesion was drawn and used as the reference. The deepest point of the cavitated lesion was recorded as the lesion depth [Shigetani et al., 2003; Waidyasoka et al., 2007]. The depth of the lesions measured from the same tooth (see above) were averaged and the mean was used for the statistical analysis (n = 3/group). Data were analyzed by one-way ANOVA and Fisher’s PLSD.

Root Surface Colonization

Sixteen bovine tooth roots were used in this assay. One slab, 18 mm × 7 mm × 3 mm, was cut from each root and sterilized by ethylene oxide. Four slabs were treated with each one of the cross-linkers (5% GA, 0.5% PA, and 0.625% GE) as described above except that they were not partially coated with nail varnish. The remaining slabs received no cross-linker treatment and served as positive controls. Slabs were placed in individual culture tubes containing 7 ml of TSB with 5% sucrose. Each tube was inoculated with equal parts (50 µl each) of overnight cultures of S. mutans and L. acidophilus. The individual slabs were removed from the culture medium and washed 3 times in 40-ml volumes of sterile deionized water and resuspended in 20 ml of deionized water. Glucose was added to a final concentration of 2.5% in deionized water, allowed to incubate at 37°C for 3 h and the pH recorded. This experiment was done in triplicate. In addition, one slab from each group was not inoculated with bacteria to serve as negative controls. These specimens were placed in deionized water, the pH of the water was measured, 2.5% glucose was added, and the final pH measured again. Means and standard deviations of final pH values were determined and compared with positive and negative controls using Student’s t test. A p value less than 0.05 was considered significant.

Results

Collagen Stability

The demineralized dentin samples treated with the cross-linkers exhibited distinct features. Without treatment with cross-linkers, demineralized dentin powder presented a white color and was suspended well in water. Although the GA group maintained a white color, the powder was precipitated much more readily in comparison to the untreated samples. Treatment with PA changed its coloration to a dark burgundy and the solubility was affected. A dark blue pigmentation was observed in the GE-treated group and the solubility was also noticeably decreased.

Based on the hydroxyproline analysis, the mean amounts of digested collagen from 2 mg of demineralized dentin matrix (n = 8) were 1,638 ± 319 and 1,416 ± 257 µg in the untreated and PBS groups, respectively. Considering the fact that collagen represents approximately 90% of the dentin matrix, these values indicate that most collagen was digested in the control groups. When treated with various cross-linkers, however, the digestibility markedly decreased to 175 ± 73, 73 ± 32, and 263 ± 159 µg in the GA-, PA-, and GE-treated groups, respectively. There was no difference in collagen digestibility among groups treated with cross-linkers and they all exhibited lower digestibility compared to the controls (p < 0.0001).

By hydroxyproline analysis, no collagen was detectable in the residues from controls, indicating that most, if not all, of the collagen had been digested. However, in the treated groups, most of the hydroxyproline was recovered in the residues. The average collagen contents in the supernatants (digested) were 12% for GA, 5% for PA and 22% for GE. When the residues were applied to SDS-PAGE, neither control groups showed discernible type I collagen on the gel. The treated groups (GA, PA, GE) showed multiple smear bands, some of which correspond to α1 and α2 chains of type I collagen (data not shown).

Root Caries Inhibition

The results of root caries inhibition assay are shown in table I and illustrated in figure 1. Lesions on the GA and PA groups were significantly different from those of the

Cross-Linkers, Collagen Degradation and Root Caries

The results for the root surface colonization assay are summarized in Table 2. The negative controls all had a pH of approximately 5.5 that did not change over the test period. Both the positive controls and the bacteria-inoculated cross-linker-treated slabs showed a significant drop in pH through 3 h of incubation with 2.5% glucose. There was no further reduction in pH through 24 h of incubation. The drop in pH of the inoculated cross-linker-treated slabs was not significantly different from that of the positive controls.

**Discussion**

In the past, a number of attempts had been made to prevent oral infectious diseases such as caries with some success [Dijkman et al., 1992; Sulkala et al., 2001; Tjaderhane et al., 1999; Waidyasekera et al., 2007]. In this study, we evaluated the efficacy of dentin collagen modification...
by naturally occurring cross-linkers in enzymatic digestibility and caries inhibition. In the case of caries, destruction of the collagen matrix in dentin requires demineralization of the tooth surface by bacteria and exposure of this matrix to proteinases derived from oral bacteria and host. The collagenolytic proteinases from bacteria act on collagen in a manner similar to that derived from the bacterium C. histolyticum, which was used in this study. These proteinases hydrolyze the peptide bond on the aminoterminal side of Gly in –X–Gly–Pro, resulting in the breakdown of collagen molecules into small peptides [Watanabe, 2004]. When the collagen molecules are cross-linked, some of the sites that serve as substrate for the collagenase could be hidden or modified due to protein folding and the enzymatic digestion can be significantly hindered [Jayakrishnan and Jameela, 1996].

The stabilization mechanisms by PA and GE may be distinct from each other. In the case of PA, though not well defined, the formation of hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl may be the primary mechanism for collagen stabilization/cross-linking [Han et al., 2003]. The decrease in collagen digestibility by PA might be due in part to its inhibition of collagenase [Koide and Daito, 1997]. In the case of GE, it has been proposed that it may polymerize first and then react with amino groups of lysine, hydroxylysine and arginine within and between collagen molecules to form intramolecular, intermolecular or intermicrofibrillar cross-links [Sung et al., 1998, 2003]. The only nonnatural cross-linker used in this study, GA, forms cross-links inter- and intramolecularly between an aldehyde and an ε-amino group of lysine and hydroxylysine of collagen. Over the last decades, GA has been widely used as a fixative for collagen-based prostheses, and in dental materials such as adhesives and desensitizers. The main drawback of GA is its toxicity. To minimize toxicity, a lower concentration is required but this results in ineffective cross-linking [Jayakrishnan and Jameela, 1996]. Both PA and GE have been used to treat a variety of tissues and have proven to be nontoxic, and the treatment increases the resistance to enzymatic digestion [Han et al., 2003; Sung et al., 1999].

No statistically significant difference was found in the amount of collagen digested after treatment with GA, PA, and GE.

When the residues were subjected to biochemical analyses, there was no detectable hydroxyproline in the controls, indicating that the collagenase used effectively digested untreated dentin collagen. However, the treated groups showed that the majority of collagen remained insoluble, i.e. undigested, and only minimal amounts of collagen were recovered in the digests, indicating that the treatment with these cross-linkers made the dentin collagen markedly more resistant to enzymatic degradation.

The staining of the dentin collagen induced by PA and GE treatment presents a clinical limitation. This effect was also observed with the root caries inhibition experiment where the treated surfaces darkened after treatment with the cross-linkers. Further studies would need to verify if this issue could be addressed by modification of the cross-linker compounds.

The effect of the natural cross-linker PA on inhibition of in vitro root caries was demonstrated in this study. For this experiment, bovine instead of human (root) dentin was used primarily because of its size, which facilitated specimen preparation and analysis. Bovine roots also yielded a higher number of specimens. A recent study has demonstrated no significant difference between these substrates in an in situ caries model [Hara et al., 2003].

Incubation of root dentin with cariogenic bacteria showed that PA treatment resulted in the inhibition of root caries lesion compared to the groups of ‘no treatment’ or GE. This could be due not only to the decrease in collagen digestibility, but also to the prevention of dentin demineralization. A few studies have reported that glutardialdehyde is capable of preventing demineralization of dentin [Arends et al., 1989; Dijkman et al., 1992]. The fixation of collagen on dentin may reduce diffusion of calcium and phosphate ions out of the dentin lesion. This is in agreement with the present study that suggests that an increase in resistance to demineralization is achieved after treatment with GA and PA. This was not likely due to attributable direct antibacterial activities, but rather to the ability of GA and PA to cross-link the dentin matrix and prevent cross-linking.

### Table 2. Effect of cross-linkers on root surface colonization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
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</thead>
<tbody>
<tr>
<td>Negative control (n = 4)</td>
<td>5.49 ± 0.03a</td>
</tr>
<tr>
<td>Positive control (n = 3)</td>
<td>4.74 ± 0.06b</td>
</tr>
<tr>
<td>GA (n = 3)</td>
<td>4.89 ± 0.07b</td>
</tr>
<tr>
<td>PA (n = 3)</td>
<td>4.61 ± 0.14b</td>
</tr>
<tr>
<td>GE (n = 3)</td>
<td>4.91 ± 0.08b</td>
</tr>
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</table>

Values represent the mean (± SD) pH of a suspension of S. mutans and L. acidophilus following incubation of slabs in 2.5% glucose for 3 h at 37°C. Negative controls are inoculated slabs and positive controls are untreated slabs. Different superscript letters represent statistically significant differences.
demineralization of the tooth structure as shown in the root surface colonization assay. Furthermore, the antibacterial effect of PA has been previously investigated against S. mutans strains and the results were negative [Leitao et al., 2005]. Although GE treatment tended to decrease collagen degradation after 72 h of treatment, it did not achieve a significant inhibition of root caries compared to that of the 'no treatment' group. This could be due to fewer cross-links and/or types of cross-links formed as a result of incubation with this cross-linker compared to PA. In addition, GE unlikely has an effect on bacteria as shown in the root surface colonization assay. Currently, we are not aware of another investigation of the antibacterial effect of GE on the oral bacteria.

To our knowledge, this is the first study demonstrating that a natural cross-linker is capable of inhibiting caries in vitro. Since PA is a natural and biocompatible compound, there is a great potential for it to be used in caries prevention.

Within the limitations of this in vitro study, it can be concluded that PA and GE could be used to modify dentin collagen to efficiently increase its resistance against enzymatic digestion. In addition, PA treatment exhibited greater prevention of root lesion formation compared to the other cross-linkers, indicating its potential use as an anti-carious reagent. The biocompatibility of PA allows such compounds to be used in vivo, which is not possible in the case of GA. Further studies are warranted to elucidate the nature of the reactions, resultant cross-links and the behavior of these components in the oral environment.

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