Genipin-modified dentin collagen:
Biochemical and biomechanical characterization

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ABSTRACT

HIROKO NAGAOKA: Genipin-modified dentin collagen:
Biochemical and mechanical characterization
(Under the direction of Dr. Mitsuo Yamauchi)

The objectives of this study were to: 1) characterize genipin (GE)-modified bovine dentin collagen treated with three different doses of GE (0.01%, 0.1% and 0.5%) for several treatment durations as related to changes in its biochemical properties, 2) evaluate changes in the biomechanical properties, as assessed by microtensile bond strength (MTBS), of human dentin collagen that had been modified by four different treatment durations of 0.5% GE. The collagen stability and collagen discoloration assays indicated that the treatment of bovine dentin collagen with GE resulted in a dose- and time-dependent decrease of collagen digestibility and increase of discoloration. Amino acid analysis indicated that the GE-induced cross-links involved the lysine and hydroxylysine residues in dentin collagen. Cross-link analysis showed that GE treatment did not affect the mature cross-link, pyrodinoline; however, dihydroxylysinonorleucine was slightly but significantly decreased ($p<0.05$) after 12 h of treatment with GE. MTBS analysis showed that MTBS significantly increased in samples treated with GE for 4 h, 12 h and 24 h when compared to control ($p<0.0001$). In addition, MTBS of samples treated with GE for 4 h was significantly higher than those of 12 h and 24 h groups ($p<0.0001$). Taken together, these results demonstrate that GE treatment improves the stability of dentin collagen and MTBS, and its potential use for caries prevention and restorative dentistry.

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To my parents Masako and Yasuhisa Nakata,

for their support, extraordinary courage and unconditional love.

To my husband Hideaki,

who constantly encouraged and supported me to make my dream reality.

To my daughter Sakura,

who has made my life wonderful, fascinating and extraordinary.

I would not have done anything without you.

I love you.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance

Arg: arginine

DDW: deionized distilled water

deH-DHLNL: dehydro-dihydroxylysinonorleucine

deH-HLNL: dehydro-hydroxylysinonorleucine

DHLNL: dihydroxylysinonorleucine

DHNL: dihydroxynorleucine

d-Pyr: deoxy-pyridinoline

EDTA: ethylenediamine-tetraacetic acid

PLSD: protected least significant difference

GA: glutaraldehyde

GE: genipin

HCL: hydrochloric acid

His: histidine

HLNL: hydroxylysinonorleucine

HPLC: high performance liquid chromatography

Hyl: hydroxylysine

Hyl^{ald}:   hydroxylysine-aldehyde

Hyp: hydroxyproline

LH: lysine-hydroxylase

LOX: lysyl oxidase

Lys: lysine

Lys^{ald}:   lysine-aldehyde

MPa: megapascal

MTBS: microtensile bond strength

NaB^3H_4:   sodium borohydride

PBS: phosphate buffered saline

PA: proanthocyanidin

Pro   proline

Pyr: pyridinoline

Introduction

Resin bonding to enamel substrate is considered a stable, predictable and reliable clinical procedure. Enamel bonding (Buonocore, 1955) relies mainly on the creation of micro-porosities by an acidic etchant on the enamel surface and subsequent penetration and micro-mechanical interlocking by a fluid adhesive resin into the prismatic and interprismatic spaces to form macro and micro-resin tags, (Swift *et al.*, 1995; Inoue *et al.*, 2002), respectively.

Bonding to dentin is more complex due to its wet tubular ultrastructure and heterogeneous, partially organic composition (Pashley, 1989; 1991). Fusayama proposed the use of phosphoric acid to remove the smear layer and demineralize the underlying dentin in order to improve adhesion (Fusayama *et al.*, 1979). Acid etching exposes superficial collagen fibrils and associated non-collagenous proteins. Dentin bonding is affected by saturating the demineralized surface with hydrophilic monomer resins, present in the adhesive systems, so as to permeate into the dentinal tubules and intertubular collagen network. The resulting combination of collagen and resin results in a mixed structure described by Nakabayashi in 1982 as the "hybrid layer" or a "resin-impregnated layer" (Nakabayashi *et al.*, 1982).

Since bonding is created by the impregnation of the dentin substrate by blends of resin monomers, the stability of the bonded interface relies on the creation of a compact and homogenous hybrid layer (Breschi *et al.*, 2008). Ideally, the adhesive monomers occupy all the space that remains following removal of the mineral by acid-etching and envelop the

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exposed collagen fibrils (Pashley *et al.*, 1993). Recent studies have shown that this objective

is frequently not achieved (Sano *et al.*, 1995; Spencer & Swafford, 1999; Spencer *et al.*,

2000; Wang & Spencer, 2002). The discrepancy between depth of dentin demineralization

after the acid-etching procedure and depth of resin infiltration allows the presence of

microporous zones underneath and in the hybrid layer detectable by silver nitrate (Sano *et al.*,

1999). These spaces may result in a pathway for degradation of the interface by hydrolysis of

collagen fibrils and/or unpolymerized resins within the hybrid layer, and/or demineralized

dentin (Sano *et al.*, 1999; Pereira *et al.*, 2001; Bedran-de-Castro *et al.*, 2004). Tay et al. has

also proposed that nanoleakage can be attributed to microvoids in which water may be

incompletely removed from the resin-dentin interfaces in etch-and-rinse and self-etch

adhesive systems (Tay *et al.*, 2002). It has been demonstrated that drying dentin after the

acid-etching step causes architectural changes of the collagen fibrils, such as collapse and

shrinkage (Kanca, 1992; Gwinnett, 1994; Van Meerbeek *et al.*, 1998). The structural changes

impair the penetration of the adhesive resulting in reduction of bond strength (Gwinnett,

1994). The introduction of the wet or moist bonding technique made maintenance of the

spatial architecture, or re-expansion of the collapsed demineralized collagen matrix possible

(Kanca, 1992).

Although significant improvements of adhesive systems have been achieved, the

bonded dentin interface remains the weakest area of tooth colored restorations (Breschi *et al.*,

2008). The durability and stability of the bonded interface remains questionable (Carrilho *et*

al., 2005; De Munck *et al.*, 2005; Tay *et al.*, 2005; Breschi *et al.*, 2008). Therefore,

improvement of dentin properties is important for effective dentin bonding.

Dentin and collagen structure

Dentin is a specialized mineralized tissue composed of 50% vol. mineral, 25% vol. organic matrix and 25% vol. fluid (Ten Cate, 1998). Dentin mineral is basically composed of carbonate-substituted hydroxyapatite. The organic matrix of dentin is composed mostly of fibrillar type I collagen (90% of the organic component) which functions as a 3-dimensional stable template for mineralization (Ten Cate, 1998; Yamauchi, 2002). The collagen molecules in the fibril are packed in parallel and staggered with respect to one another by a period of ~67 nm that creates a unique periodic pattern of the fibril at the ultrastructural level (Yamauchi, 2002). The period may vary according to the degree of hydration of the tissue, decreasing from 67 to 64 nm in dehydrated samples (Baer *et al.*, 1988; Bella *et al.*, 1995; Wess & Orgel, 2000). Type I collagen molecule is a heterotrimeric molecule composed of two α1(I) chains and one α2(I) chain. The molecule contains three structural domains: N-nontriple helical (N-telopeptide), triple helical, and C-nontriple helical domains (C-telopeptide) (Yamauchi, 2002). The triple helical domain represents more than 95% of the molecule composed of repetitive amino acid sequence of glycine (Gly)-X-Y where X is frequently proline (Pro) and Y hydroxyproline (Hyp). Both N- and C- terminal nontriple helical domains contain lysyl/hydroxylysyl residues which can be enzymatically converted to the respective aldehydes to initiate the process of covalent intermolecular cross-linking (Yamauchi, 2002). The collagen biosynthesis is a long complicated process (Figure A). After gene transcription and translation of mRNA, the pro α chains (pro α1 and pro α2) acquire a number of post-translational modifications that take place in- and outside of cells. Those modifications include hydroxylation of specific Pro and lysine (Lys) residues to form Hyp and hydroxylysine (Hyl). The content of Hyp in type I collagen is relatively high,

representing about 10% of the total amino acids. The lysine hydroxylation occurs at the X-Lys-Gly sequence that is located in the triple helical domain and also at both N- and C-telopeptide domains of the collagen molecule (Gerriets *et al.*, 1993). Another intracellular post-translational modification is the glycosylation of specific Hyl residues to form galactosyl- or glucosyl-galactosyl Hyl. The formation of Hyp is important for stabilization of the triple helix structure and hydroxylysine for glycosylation and cross-linking. The glycosylation of hydroxylysine is thought to regulate cross-linking maturation (Yamauchi *et al.*, 1986; Sricholpech *et al.*, 2012; Yamauchi & Sricholpech, 2012), collagen fibrillogenesis and remodeling (Notbohm *et al.*, 1999). The covalent intermolecular cross-links that tie collagen molecules are responsible for the maintenance of the integrity and stability of collagen fibrils. Post-translational modifications, particularly Lys hydroxylation and cross-linking, are tissue-specific and vary significantly depending on the tissue's physiological/pathological conditions (Brodsky *et al.*, 1980; Yamauchi & Katz, 1993)

Type I collagen provides tissues and organs with tensile strength, form and connectivity (Sung *et al.*, 1999; Ritter *et al.*, 2001; Bedran-Russo *et al.*, 2007). The collagen molecules in a fibril are stabilized by lysyl oxidase-mediated covalent intermolecular cross-linking (Rivera & Yamauchi, 1993; Yamauchi & Shiiba, 2002) and this process is critical for the formation of stable, functional fibrils.

Collagen cross-linking

The major cross-linking pathway of dentin collagen is the Hyl aldehyde (Hyl [ald])-derived pathway (Figure B). In the Hyl [ald] pathway, the Hyl [ald] may pair with Hyl, forming an aldimine cross-link, dehydroxylysinonoleucine (deH-DHLNL) or with Lys forming dehydro-hydroxylysinonoleucine (deH-HLNL) (Figure C). These cross-links are reducible with

sodium borohydride (NaBH$_4$) cross-links and may mature into more complex, non-reducible trivalent cross-links, pyridinoline (Pyr) (Figure D) and pyrrolic compounds. Pyr is a naturally fluorescent cross-link and likely a maturational product of deH-DHLNL The deH-DHLNL is relatively stable in the tissue and this is due likely to a spontaneous Amandori rearrangement of the aldimine bond to ketoamine, thus accounting for the large extent of the insolubility of dentin collagen (Cagidiaco & Ferrari, 1995).

The patterns of cross-linking, including the pathways, the concentration of the individual cross-links, and their molecular distributions, are tissue specific and are probably related to the physiological functions of each tissue, but not to the particular genetic types of collagen (Eyre *et al.*, 1984; Yamauchi & Mechanic, 1988). Even within the same tissue, the cross-linking to some extent varies depending on sites within the tissue (Yamauchi *et al.*, 1991). The tissue-specific cross-linking pattern is likely regulated by expression and activity of various lysyl hydroxylase (LH) and lysyl oxidase isoforms in specific cell types.

Intra- and inter-tooth variations of the amounts of collagen cross-links in dentin have been reported (Rivera & Yamauchi, 1993; Miguez *et al.*, 2004). Rivera and Yamauchi (Rivera & Yamauchi, 1993) reported significant increase in the content of Pyr, a small increase in the content of DHLNL, and minute increases in the content of deoxy-pyridinoline (d-Pyr) or HLNL from incisors to canine-premolars, and to molar, respectively. Miguez *et al* (Miguez *et al.*, 2004) reported significant differences in the amount of cross-links between crown and root dentin of human molars. Collagen content in crown and root was comparable to each other, however, the amounts of DHLNL and Pyr cross-links were found significantly higher in root dentin than those of crown dentin.

Cross-linking agents

It has now been well documented that the stability of the collagen fibrils can be further enhanced by the treatment with chemical- as well as natural cross-linkers (Walter *et al.*, 2008; Macedo *et al.*, 2009).

One of the most widely used chemical cross-linkers in dentistry is glutaraldehyde (GA); however, its use in clinical settings is limited due to its toxicity (Lohre *et al.*, 1992; Lohre *et al.*, 1993; Nishi *et al.*, 1995). Considering this limitation, the potential use of natural plant and fruit-derived cross-linking agents such as proanthocyanidin (PA) (Fig. E) and genipin (GE) (Fig. F) has recently been explored (Bedran-Russo *et al.*, 2007; Walter *et al.*, 2008; Macedo *et al.*, 2009). These studies demonstrated that both compounds effectively stabilize dentin collagen and improve dentin bond strength.

Of these two compounds, PA has been characterized for its effects on fibroblasts (Han *et al.*, 2003) and chemical stability of dentin collagen (Walter *et al.*, 2008; Macedo *et al.*, 2009). It has been proposed that PA-treatment could enhance the dentin collagen stability, and improve the ultimate tensile strength in dentin collagen (Bedran-Russo *et al.*, 2007). However, it has been reported that the treatment with PA caused discoloration on demineralized dentin samples (Walter et al., 2008).

GE is a compound derived from genoposide, which is isolated from the fruits of Gardenia jasminoides (Sung *et al.*, 1999; Sung *et al.*, 1999; Fujikawa *et al.*, 1976). It was found that GE-fixed tissue had a resistance against enzymatic degradation comparable to or better than GA-fixed tissue and GE is 1000 times less cytotoxic than GA (Sung *et al.*, 1999; Sung *et al.*, 2001). Sung et al, found that GE can react with the amino groups of amino acids such as Lys, Hyl, and Arg residues within biological tissues (Sung *et al.*, 1998); however, in

comparison to PA, the effects of GE on dentin are still poorly characterized and the mechanism of GE-induced cross-linking is still not well understood.

The aims of the current study are to characterize GE-modified dentin collagen by evaluating the collagen stability, amino acid composition, lysyl oxidase (LOX)-mediated collagen cross-links, and microtensile bond strength (MTBS). A better understanding of the mechanism of GE modification on dentin collagen may provide insight into a development of new approaches to prevent dentin caries, and to improve dentin bond strength and longevity of the resin-dentin bond established during restorative procedures.

Figure A.

Collagen Biosynthesis.

Schematic for the biosynthesis of type I collagen. The upper part (above the cell membrane) summarizes the intracellular and the lower (below the cell membrane) the extracellular events. The intracellular events include extensive post-translational modifications such as hydroxylation, glycosylation (both O- and N-linked), association of pro α chains and folding into a triple helical molecule from the C- to N-terminus. The extracellular events involve the removal of both N- and C-propeptide extensions, self-assembly of collagen molecules into a fibril, enzymatic oxidative deamination of Lys and Hyl residues by LOX and subsequent intra- and intermolecular covalent cross-linking. The collagen molecules are packed in parallel and are longitudinally staggered with respect to one another by some multiple of axial repeat distance, D (~67nm).

Collagen Biosynthesis
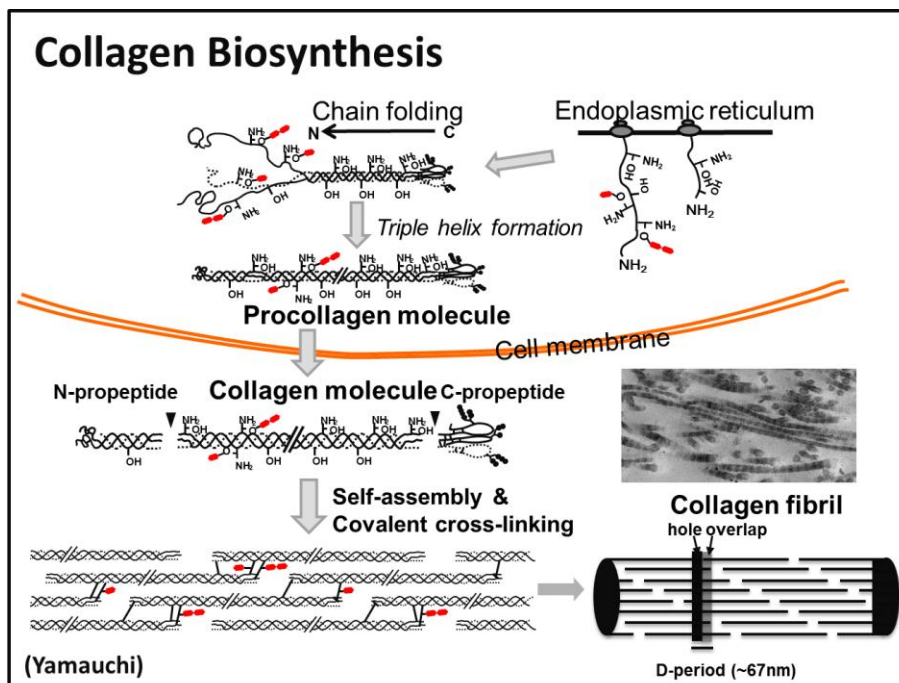
Figure B.

Major cross-linking pathways of collagen.

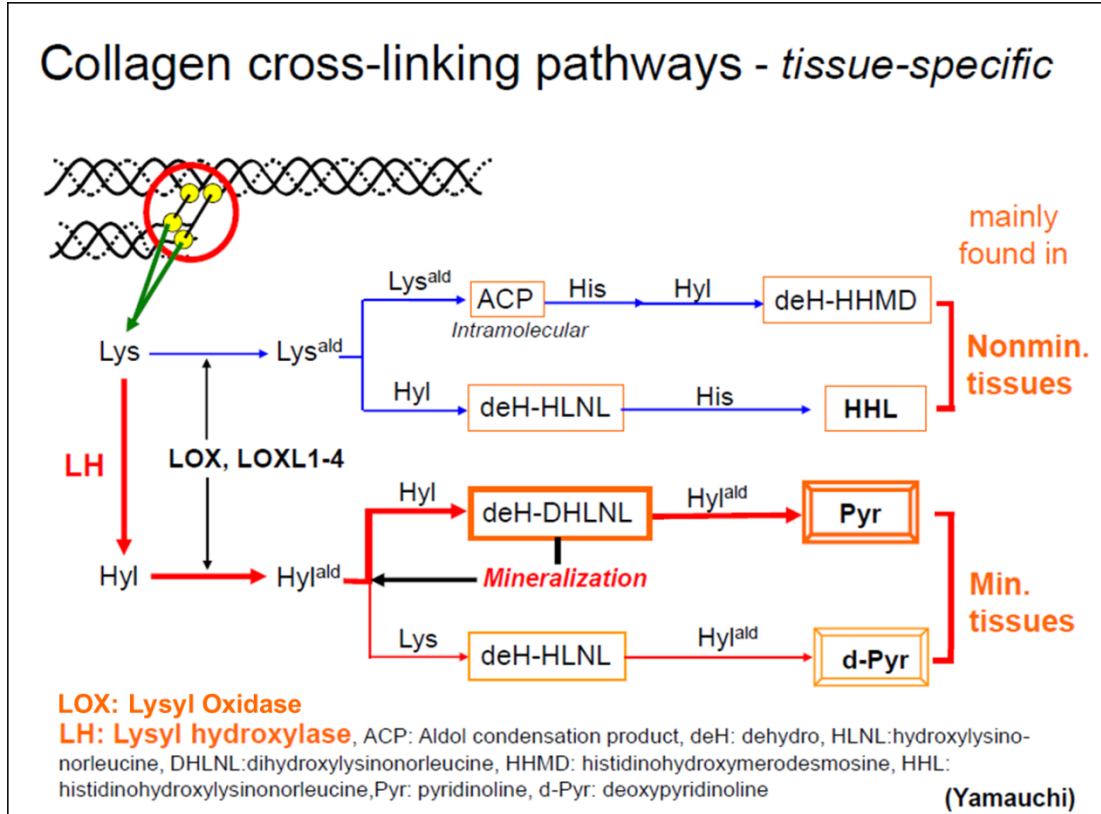Figure C.

Structure of dehydrodihydroxylysinonorleucine (deH-DHLNL).

When Hyl[ald] in the C- or the N-telopeptides pairs with helical Hyl, an iminium cross-link, deH-DHLNL is formed.
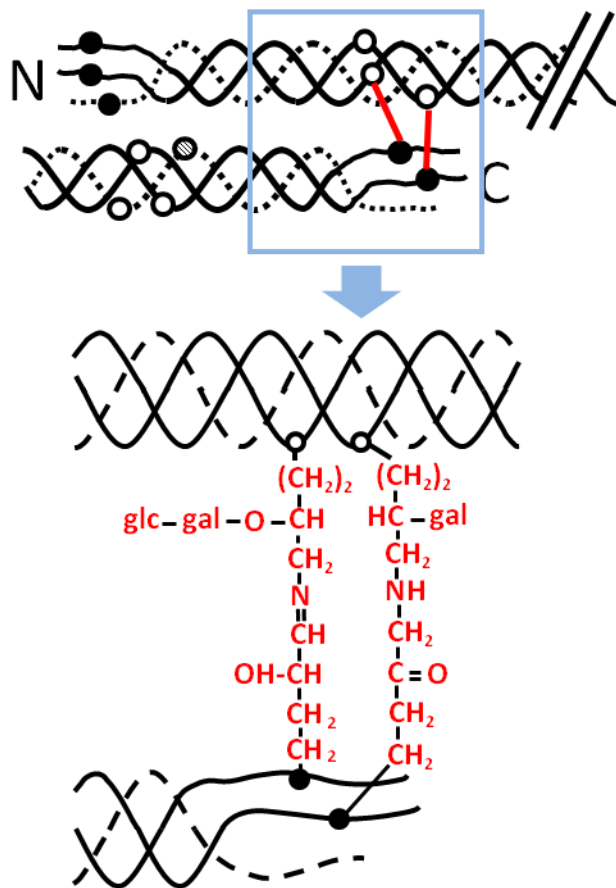
Figure D.

Structure of pyridinoline (Pyr).

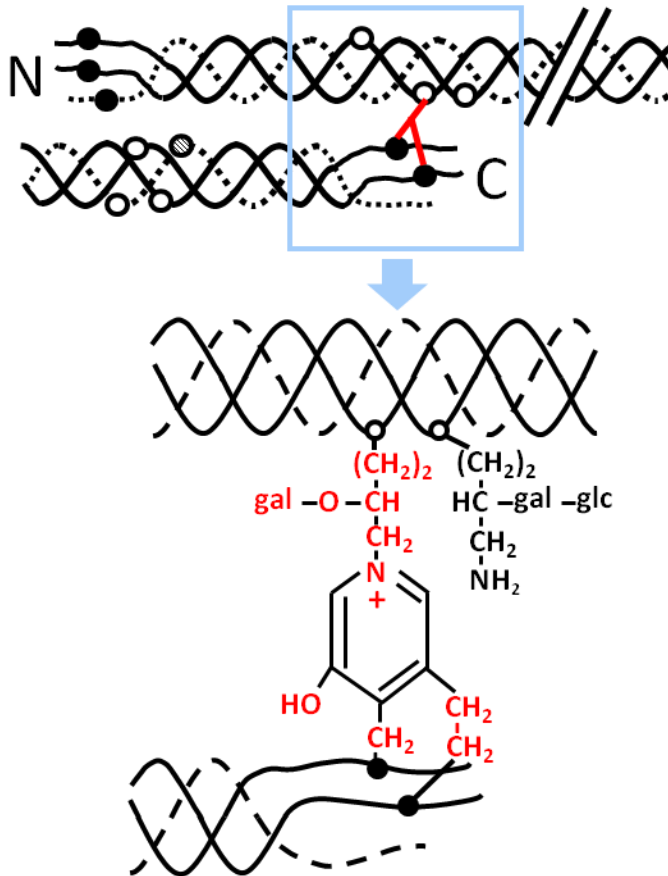Pyr is derived from a helical Hyl and two Hyl^{ald} in the C- or the N-telopeptides.

Figure E. Structure of Proanthocyanidine   Figure F. Structure of Genipin
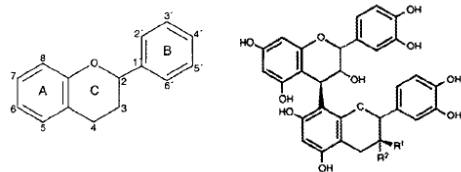
<center>**Chapter I**</center>

<center>**Characterization of genipin-modified dentin collagen Introduction**</center>

Introduction

Fibrillar type I collagen is the predominant component in dentin matrix, and regulate the spatial aspect of dentin formation. For instance, it functions as a stable, three-dimensional template for mineral deposition and growth and it also defines the space for organized mineralization (Parsons & Glimcher, 1976). Cross-linking is the final post-translational modification in collagen biosynthesis and is crucial for the stability, tensile strength, viscoelasticity and appropriate physiological functions of the collagen fibrils (Nimni & Harkness, 1988; Yamauchi & Mechanic, 1988; Kagan, 2000; Yamauchi, 2002). Collagen cross-linking can be divided into two categories: enzymatic lysyl oxidase (LOX) mediated cross-links, and non-enzymatic cross-links induced by chemical and natural agents, UV light, and heat.

LOX-mediated collagen cross-linking has been extensively reviewed (Tanzer, 1976; Eyre *et al.*, 1984; Yamauchi & Mechanic, 1988; Yamauchi, 1996; Bailey, 2001). The chemical structure and the quantity of these cross-links are primarily determined by the extent of hydroxylation of lysine (Lys) residues in the collagen molecule and the extent of oxidative deamination of the Lys and hydroxylysine (Hyl) residues present in the telopeptide

domains of the molecule. These modifications are catalyzed by lysyl hydroxylases (LHs) and LOX/LOX-like proteins, The glycosylation pattern of specific Hyl residues that are involved in cross-linking may regulate the maturation of collagen cross-links (Sricholpech *et al.*, 2012). The cross-linking pattern can also be determined by the maturation/turnover rate of tissues (Bailey & Shimokomaki, 1971; Moriguchi & Fujimoto, 1978; Eyre *et al.*, 1988; Yamauchi *et al.*, 1988), the details of molecular packing structure (Yamauchi *et al.*, 1986; Mechanic *et al.*, 1987; Katz & David, 1992), and the physical force exerted on the tissue (Otsubo *et al.*, 1992).

Collagen cross-links can also be induced non-enzymatically by the treatment with chemicals and natural plant/fruit extracts (Sung *et al.*, 1999). These non-enzymatic cross-linking has been shown to facilitate preservation of substrate shape as a proper scaffolds (Sung *et al.*, 2001), improve a tissue's physiological function (Sung *et al.*, 1999; Chang *et al.*, 2002; Liang *et al.*, 2004), and increase the mechanical properties of collagen (Huang *et al.*, 1998; Sung *et al.*, 1999; Han *et al.*, 2003) (Walter *et al.*, 2008). Glutaraldehyde (GA), a synthetic cross-linking agent, has been widely used as a fixative agent and it is well documented that GA treatment improves the stability of various collagen-based tissues (Chang *et al.*, 2002; Bedran-Russo *et al.*, 2008; Bedran-Russo *et al.*, 2011; Braile *et al.*, 2011). However, the utilization of GA on biological tissues *in vivo* has been limited because of its toxicity and/or instability over time (Sung *et al.*, 1998; Sung *et al.*, 1999).

Genipin (GE), a traditional Chinese herbal medicine extracted from gardenia fruit, has been found to be an effective collagen cross-linking agent (Fig. 1.1). GE has favorable *in vitro* cytotoxicity and the GE-modified collagenous tissues have increased toughness more than that of GA-treated collagenous tissues (Cheung *et al.*, 1985; Tsai *et al.*, 2000).

While several studies on the effects of GE on dentin have been published (Bedran-Russo *et al.*, 2007; Walter *et al.*, 2008; Al-Ammar *et al.*, 2009), conditions of GE treatment are not well established and the nature of GE-induced cross-linking is still not well understood. A better understanding of GE-induced cross-links in dentin collagen could provide insight into a strategy to develop a novel biomodification technology in restorative dentistry. As the first step toward this goal, the present study was designed to characterize GE-modified dentin collagen matrices using various treatment conditions. Specifically, discoloration of dentin, collagen stability against bacterial collagenase and changes in amino acid composition and LOX-mediated collagen cross-links were analyzed.

Materials and methods

Sample collection and demineralization

Extracted intact bovine molars (≤1 year old) were purchased (Aries Scientific, Texas, USA) and used for this study. Enamel, cementum and pulp were removed using high-speed diamond burs under water cooling. Dentin was pulverized in liquid N_2 by a Spex Freezer Mill (SPEX CertiPrep, Inc., Metuchen, NJ, USA), washed with distilled water. In order to obtain sufficient quantities of collagen for biochemical and statistical analyses, three teeth were combined as one group. The samples were demineralized with 0.5 M ethylenediaminetetraacetic acid (pH 7.4) (EDTA) with several changes of the solution for 14 days at 4 °C, extensively washed with distilled water and lyophilized. Eighty four 2 mg aliquots of the demineralized dentin collagen were randomly allocated to the following four treatment groups based on the GE (Wako Pure Chemical Industries, Ltd., Osaka, Japan) concentrations (n=21):

1. Control group. In the control group, specimens were treated with phosphate buffered saline (PBS);

2. 0.01% GE. In this group, specimens were treated with 0.01% GE in PBS;

3. 0.1% GE. In this group, specimens were treated with 0.1% GE in PBS; and

4. 0.5% GE. In this group, specimens were treated with 0.5% GE in PBS.

Discoloration and collagen stability of GE-modified dentin collagen

Seventy two 2 mg aliquots of demineralized dentin collagen were treated with 1 mL of three concentrations of GE solution or PBS and incubated for 30 min, 1 h, 4 h, 8 h, 12 h and 24 h at 37 °C with agitation (n=3 in each time point, n=18 in each group). After treatment, the samples were extensively washed with distilled water, lyophilized and observed for their discoloration.

Collagen stability was assessed by enzymatic degradation as reported (Walter *et al.*, 2008). Samples were suspended in 1 mL of ammonium bicarbonate and digested with 5% w/w bacterial collagenase derived from *Clostridium histolyticum* (Worthington Biochemical Corp., Lakewood, NJ, USA, 1,075 U/ mg) for 24 h at 37 °C. After digestion, the samples were centrifuged for 15 min at 15,000 g, the residues (undigested collagen) were washed with distilled water by repeated centrifugation and lyophilized. The residues were hydrolyzed with 300 μL of 6 N HCl (Pierce, Rockford, IL, USA) *in vacuo* after flushing with N_2 gas for 22 h at 105°C. The hydrolysates were dried, reconstituted in 300 μL of distilled water, filtered and subjected to hydroxyproline (Hyp) analysis using a high-performance liquid chromatography (HPLC) system (Prostar 240/310, Varian, Walnut Creek, CA, USA)

fitted with a cation exchange column (AA911; Transgenomic, Inc., San Jose, CA, USA) (Yamauchi & Shiiba, 2008).**Amino acid analysis and collagen cross-link analysis**

Twelve 2 mg aliquots of demineralized dentin collagen were treated with three concentrations of GE solution or PBS and incubated for 24 h at 37 °C (n=3, Total sample n=12). After treatment, the samples were extensively washed with distilled water, lyophilized, reduced with standard NaB^3H_4 and hydrolyzed and an aliquot of each hydrolysate was subjected to amino acid analysis as described above. The amount of each amino acid was calculated as residues per 1,000 total amino acids. The hydrolysates with known amounts of Hyp were then analyzed for cross-links as we reported (Yamauchi & Shiiba, 2008). The reducible immature cross-link, dehydrodihydroxylysinonorleucine (deH-DHLNL) (Fig. 1.2A)/its ketoamine (Fig. 1.2B), was analyzed as its reduced form, i.e. dihydroxylysinonorleucine (DHLNL). The non-reducible mature cross-link, pyridinoline (Pyr) (Fig. 1.2C.), was also analyzed simultaneously. The DHLNL and Pyr were quantified as moles/mole of collagen based on the value of 300 residues of Hyp per collagen. All analyses were done in triplicate in independent experiments.

Statistical analysis

The statistical evaluations were performed using Stat View software (SAS Institute Inc., Cary, NC, USA). Values were expressed as mean +/- standard deviation, and the difference between the control group and GE groups was compared by two-way ANOVA and Fisher's PLSD. A p value of less than 0.05 was considered significant.

Results

Discoloration and collagen stability of GE-modified dentin collagen

The demineralized dentin collagen treated with GE exhibited distinct features. While dentin collagen in the control group presented a white color, a dark blue pigmentation was observed in the GE-treated groups (Fig. 1.3). Hyp analysis showed that dentin collagen in all of the control groups was almost completely digested. However, when treated with GE, the digestibility markedly decreased in a dose- and time-dependent manner. In the 0.5% GE, 81.9 ± 4.9 nM, 166.4 ± 3.8 nM, 926.6 ± 37.6 nM, $1,243.5 \pm 50.6$ nM, $1,259.1 \pm 48.3$ nM and $1,259.3 \pm 61.4$ nM Hyp at 30 min, 1 h, 4 h, 8 h, 12 h and 24 h were recovered in the undigested residue respectively. In the 24 h treatment of PBS, 0.01% GE and 0.1% GE, 2.0 ± 1.7 nM, 166.0 ± 19.3 nM and $1,054.3 \pm 47.3$ nM Hyp were recovered in the undigested residues (Fig.1.4).

Amino acid Analysis

Overall amino acid compositions in the control and three GE groups at all time points examined were essentially identical to one another (data not shown) except Hyl and Lys. The amounts of amino acids, Hyl and Lys in control and three GE groups treated for 24 h are shown in Table 1. Hyl and Lys in three GE groups were significantly decreased in a dose-dependent manner when compared to the control ($p<0.05$). No significant changes were observed for other amino acids including arginine, which is different from the report by Sung and co-workers (Sung *et al.*, 1998).

Collagen cross-link analysis

Typical chromatographic profiles of collagen cross-links from control and three GE groups treated for 24 h are shown in Fig. 1.5 & 1.6 Two major cross-links, DHLNL (reducible) and Pyr (non-reducible) were identified in all groups at all time points examined. In addition, unknown newly formed NaB^3H_4-reducible compunds were identified in three GE groups (peaks at 22 min: unk 1, and at 64 min: unk 2) (Fig.1.5). The results of the quantitative cross-link analysis comparing control group with GE groups are summarized in Table 1.2.

The amount of DHLNL in 0.01% and 0.1% GE for 24 h (1.35 ± 0.23 M and 1.23 ± 0.19 M, respectively) were not significantly different from those of control (1.36 ± 0.17 M), but DHLNL in 0.5% GE for 24 h (1.13 ± 0.07 M) was slightly but significantly decreased when compared to that of control ($p < 0.05$) (Fig. 1.5 and Table 1.2). The amount of Pyr in all three GE groups for 24 h showed no significant difference when compared with control (Fig. 1.6, and Table 1.2).

The amounts of newly formed reducible compounds, unk 1 and unk 2, in GE groups for 24 h were significantly increased in a dose-dependent manner (Fig.1.5 and Table 1.2).

Discussion

Fibrillar type I collagen is the most abundant protein and the major structural component in dentin and the molecules in the fibril are stabilized by covalent intermolecular cross-linking

To further stabilize collagen fibrils in biological tissues, various cross-linking agents have been used to induce additional intra- and intermolecular cross-links (Cheung *et al.*, 1985; Huang *et al.*, 1998; Sung *et al.*, 1998; Sung *et al.*, 1999; 1999; Sung *et al.*, 1999; Tsai *et al.*,

2000; Sung *et al.*, 2001; Chang *et al.*, 2002; Han *et al.*, 2003; Liang *et al.*, 2004; Bedran-Russo *et al.*, 2007; Bedran-Russo *et al.*, 2008; Walter *et al.*, 2008; Al-Ammar *et al.*, 2009; Bedran-Russo *et al.*, 2011; Braile *et al.*, 2011). GE, a naturally occurring cross-linking agent, is as efficient as GA but possesses significantly less toxicity (Sung *et al.*, 1998; Sung *et al.*, 1999) and has been proven to be biocompatible and stable for a long period of time in animals (Sung *et al.*, 1999). Although several cross-linking agents including GE have been proposed to improve the mechanical and chemical properties of collagenous tissues, the mechanisms of cross-linking and the nature of the cross-links are still not well defined.

The present study was undertaken to evaluate the effects of GE on dentin collagen using biochemical approaches such as Hyp analysis, amino acid analysis and quantitative cross-link analysis. GE-treatment of collagen resulted in a blue pigmentation as previously reported (Sung *et al.*, 1998; Lee *et al.*, 2003; Walter *et al.*, 2008). The blue discoloration of demineralized dentin collagen by GE increases in a dose- and time-dependent manner. The mechanism of the blue pigmentation is not yet clear but possibly associated with GE-induced cross-link compounds as the intensity of the color well corresponded with the collagen stability against enzymatic digestion. Collagen stability was evaluated by the digestibility with bacterial collagenase which hydrolyzes the peptide bond on the amino-terminal side of Gly in –X–Gly–Pro. This results in the breakdown of collagen molecules into small peptides (Watanabe, 2004). Based on the mean values of Hyp in 2 mg demineralized dentin matrix in this study (1,319.4 $\pm$ 46.9 nM) (n= 3), the rate of collagen digestion was 93.8%, 87.4%, 27.3%, 7.6%, 1.1% and 1.0% in 0.5% GE at 30 min, 1 h, 4 h, 12 h and 24 h respectively, and 99.7%, 89.0% and 16.0% in PBS, 0.01% GE and 0.1% GE for 24 h respectively. Thus, the rate of collagen digestion with GE treatment cleary decreased in a dose- and time-dependent manner (Fig. 1.4). When collagen was treated with 0.5% GE for 12 h and 24 h, almost no

collagen was digested. Most likely GE-induced cross-linking hinders the enzyme accessibility to collagen and/or it generates a large cross-linked collagen complex so that collagenase cleavage no longer solubilizes the complex.

The amino acid analysis was conducted to determine which, if any, amino acid residues in the dentin collagen reacted with GE. The results demonstrated that Hyl and Lys residues were the only amino acids that decreased with GE treatment in a dose- and time-dependent manner. It is likely that the ε-amino groups on Hyl and Lys in a collagen molecule react with GE to form cross-links that are stable in acid hydrolysis. When the values of Lys and Hyl were calculated on a residues per collagen molecule basis (based on the value of 300 residues of Hyp per collagen molecule), the mean number of Hyl residues in GE groups for 24 h per collagen molecule decreased from 47.7 ($\pm$ 3.2) in control to 5.3 ($\pm$ 1.4) in 0.5% GE group respectively. In case of Lys, it decreased from 97.9 ($\pm$ 13.2) in control to 7.6 ($\pm$ 5.1) in 0.5% GE group, respectively. It is estimated that approximately 90 Lys and 42 Hyl in a collagen molecule are utilized for GE-induced cross-links in 0.5% GE treatment for 24 h. Since there are approximately 98 Lys and 48 Hyl residues per collagen molecule in controls (note: some of the Lys residues are derived from non-collagenous proteins), ~92% of the Lys and 88% of the Hyl residues of collagen were likely involved in GE-derived cross-linking with 0.5% GE treatment for 24 h. Under the conditions used, no other amino acids were significantly changed by GE treatment. Sung et al reported that Lys, Hyl and Arg were used in the reaction with GE in porcine pericardia (Sung *et al.*, 1998). The discrepancy between the current study and the one by Sung et al., could be due to the different treatment conditions and/or tissues.

Cross-link analysis revealed that several unidentified, reducible compounds were detected in GE-treated groups; two major ones eluted at 22 and 64 min in our HPLC system,

respectively, and they increased in a dose- and time-dependent manner. These newly formed two reducible compounds are most likely GE-induced cross-links that involve Lys and Hyl residues in collagen. Further studies are warranted to identify the structures of these cross-links by isolating and analyzing mass spectrometry and NMR. There was a significant decrease of DHLNL in 0.5% GE group for 24 h when compared to that of control. The cause of this slight decrease is not clear but, possibly, the aldimine bond of deH-DHLNL could be modified when dentin collagen is treated with GE for a longer period of time. Pyr showed no significant changes at all time points examined and any dose of GE used. This is likely due to the stability of this cross-link.

Conclusions

The treatment of bovine dentin collagen with GE resulted in a dose- and time-dependent increase in collagen stability and the intensity of the discoloration. The GE-induced, reducible cross-links involving Lys and Hyl residues also increase in a time- dose-dependent manner. The GE treatment does not affect LOX-mediated collagen cross-link, Pyr, but could modify some of DHLNL cross-link.

Table 1.1

Contents of hydroxylysine, lysine.

	Control	0.01% GE	0.1% GE	0.5% GE
Hyl	12.8±0.8	7.5±1.8[**]	4.3±0.3[***,a]	1.6±0.4[***,b,†]
Lys	26.2±3.5	18.8±0.8[*]	9.8±2.3[***,b]	2.3±1.6[***,c,††]

The average values and SDs are shown as relative amounts in 1,000 total residues. (means ± S.D.). Hyl; hydroxylysine, Lys; lysine. n=3.

*p<0.05 different from the value of control
**p<0.005 different from the value of control
***p<0.001 different from the value of control
[a]p<0.05 different from the value of 0.01% GE
[b]p<0.005 different from the value of 0.01% GE
[c]p<0.001 different from the value of 0.01% GE
†p<0.05 different from the value of 0.1% GE
††p<0.01 different from the value of 0.1% GE

Table 1.2

Contents of enzymatic cross-links and GE-induced cross-links on bovine dentin

	Control	0.01%GE	0.1%GE	0.5%GE
DHLNL	1.36 A ±0.17	1.35 A ±0.23	1.23 A ±0.19	1.13±0.07 [*]
Pyr	0.190 B ±0.085	0.188 B ±0.014	0.187 B ±0.084	0.186 B ±0.083
unk 1 (22min)	3169 C ±793	26991 C ±4007	110483 D ±6525 [*,c]	142893 D ±25133 [***,b]
unk 2 (64 min)	1787 E ±90	29160 E ±8418	136342 F ±21531 [**,a]	253668 F ±45338 [**,b]

All values in DHLNL and Pyr are expressed in moles/mole collagen (means ± S.D.).
All values in unk 1 and 2 are expressed in DPM (means ± S.D.).
DHLNL, dihydroxylysinonorleucine; Pyr, pyridinoline; GE, genipin. n=3.

*p<0.05 different from the value of control
**p<0.01 different from the value of control
***p<0.001 different from the value of control
[a] p<0.05 different from the value of 0.01% GE
[b] p<0.005 different from the value of 0.01% GE
[c] p<0.001 different from the value of 0.01% GE
Values with same letters are not statistically significant (p> 0.05).

Figure 1.1

Chemical structure of GE in the study

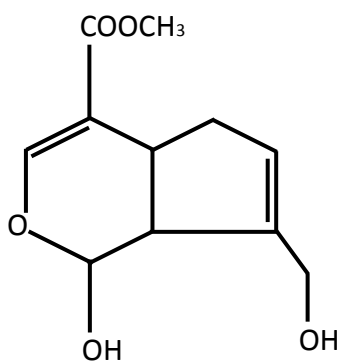Figure 1.2A.B.C

Chemical structure of iminium (A) and keto (B) form deH-DHLNL, and Pyr (C) among the type l collagen molecules. Solid line: α1 chains of type l collagen. Dotted line: α2 chain of type l collagen. Black dot: Hyl^{ald}. White dot: Hyl. glc: glucose. gal: galactose.

Fig.1.2A. Iminium form of deH-DHLNL
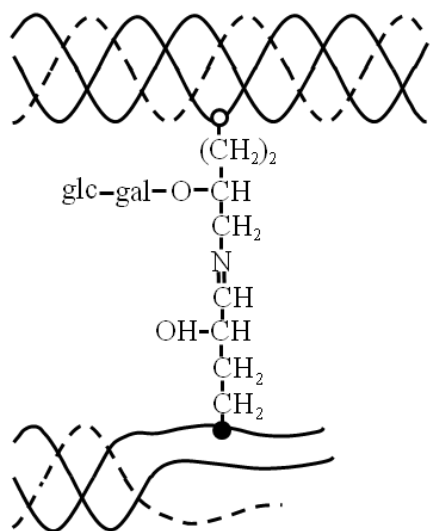
Fig.1.2B. Keto form of deH-DHLNL
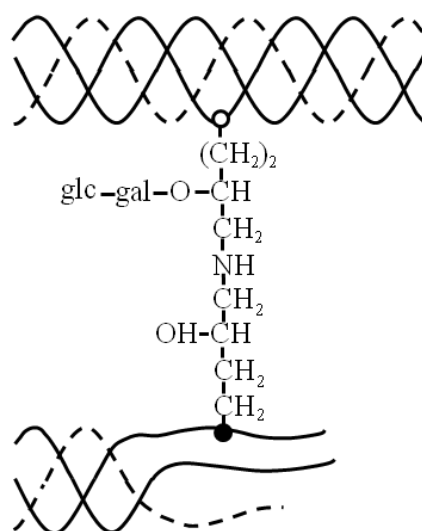
Fig.1.2C. Pyr
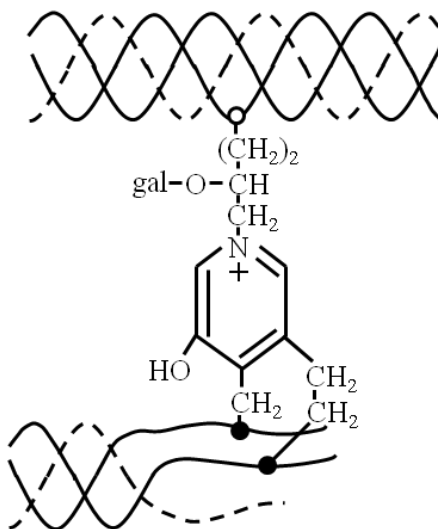
Figure 1.3

Discoloration of GE-modified dentin collagen. Photographs of the representative

discoloration of dentin collagen treated with PBS or three different concentrations (0.01%,

0.1% and 0.5%) of GE for six treatment durations (30 min, 1 h, 4 h, 8 h, 12 h, and 24 h).
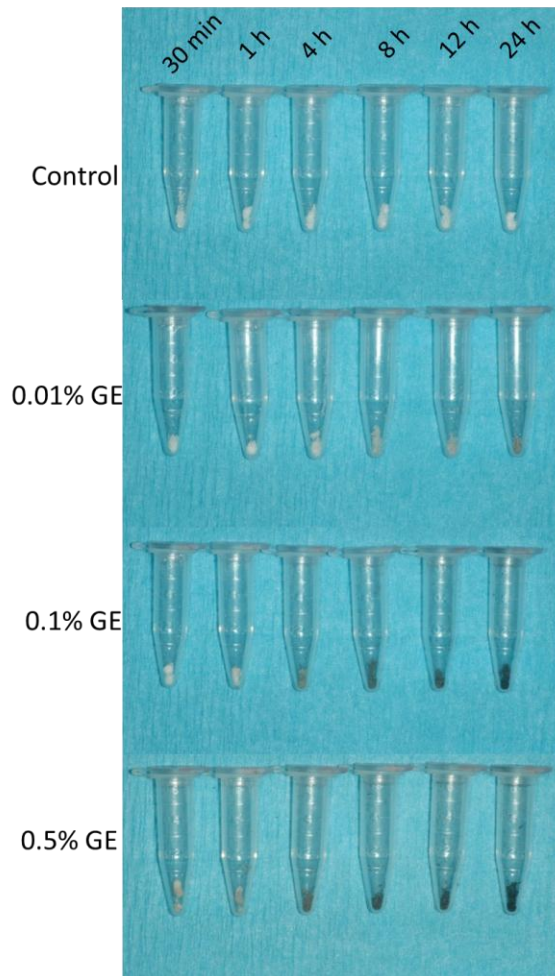
Figure 1.4

Collagen stability of GE-modified dentin collagen. The mean amounts of undigested collagen
from 2mg of dentin collagen treated with PBS or three concentrations of GE (0.01%, 0.1%
and 0.5%) for six treatment durations (30 min, 1 h, 4 h, 8 h, 12 h, and 24 h) as showing
hydroxyproline residue amounts in residue after collagenase digestion for 24 h (n=3).
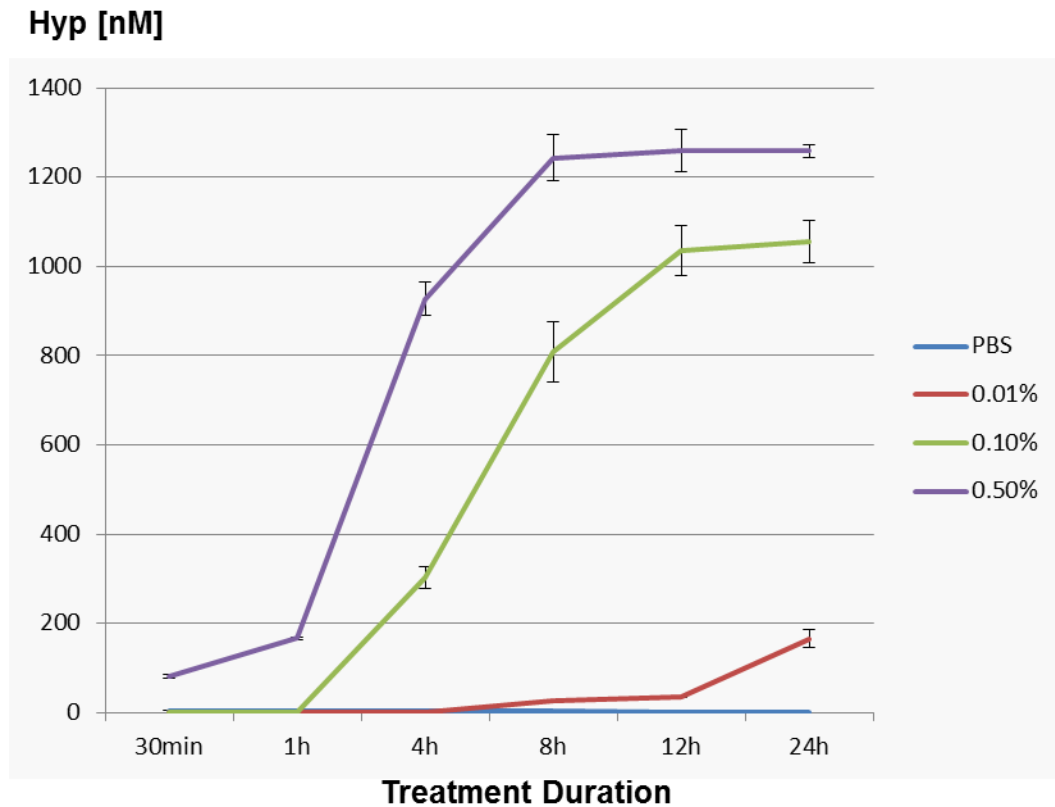
Figure 1.5

Representative chromatographs of reducible collagen cross-links in dentin collagen at 24h
treatment of PBS or three concentrations of GE (0.01%, 0.1% and 0.5%).
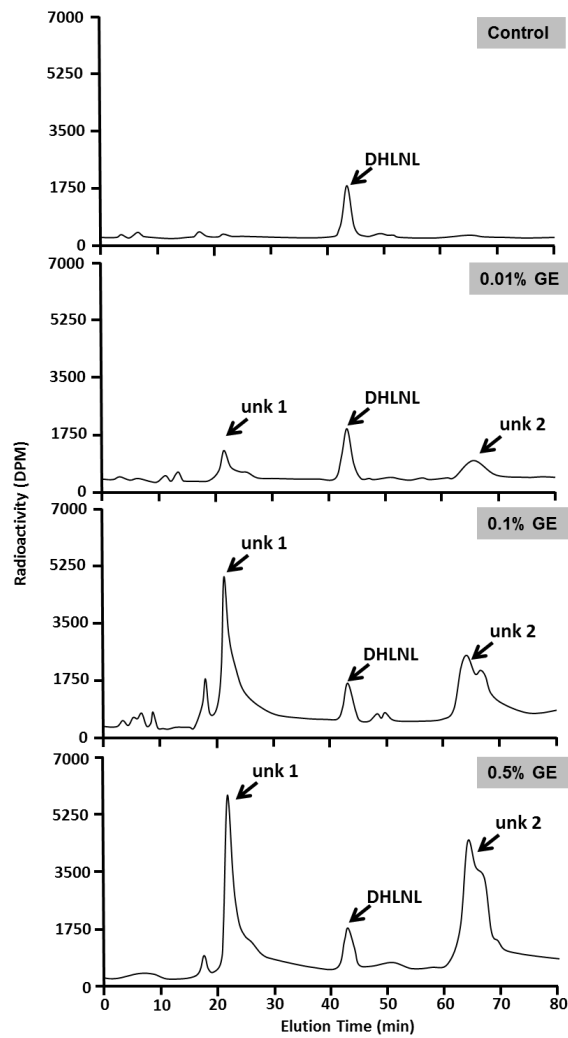
Figure 1.6

Representative chromatographs of non-reducible collagen cross-links in dentin collagen at 24h treatment of PBS or three concentrations of GE (0.01%, 0.1% and 0.5%).
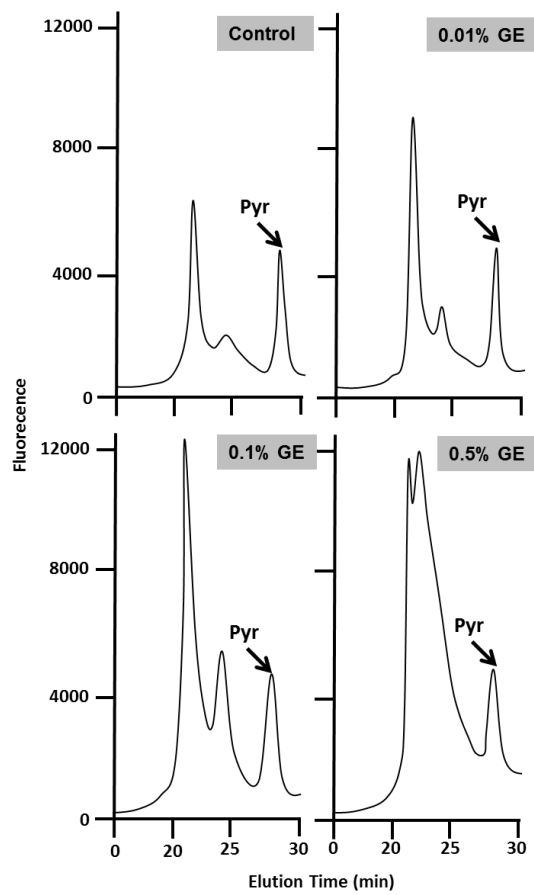
Chapter II

Genipin-induced Cross-linking and Dentin Bonding in Human Dentin

Introduction

While composite resin bonding to enamel substrate has been shown to be reliable, bonding to dentin substrate remains a challenge (Swift *et al.*, 1995). Current adhesive systems bond to dentin through a micromechanical mechanism relies on the formation of a hybrid layer (Burrow *et al.*, 1994). Hybrid layer formation can be summarized in the following steps: 1) dentin is etched prior to or simultaneous with the application of a primer/adhesive system which results in the exposure of superficial collagen fibrils, 2) adhesive resin monomers are then polymerized among and around the exposed collagen fibrils forming a hybrid layer that is believed to be essential for dentin bonding. Therefore, for effective bonding, the stability and maintenance of dentin collagen is very important (Hashimoto *et al.*, 2003; Breschi *et al.*, 2008).

Dentin is composed of an extracellular matrix embedded in a predominant inorganic mineral phase. Type I collagen is the most abundant organic component in dentin, comprising approximately 90% of the total organic matrix (Butler, 1995; Beateman *et al.*, 1996). Collagen molecules are stabilized in fibrils by the extensive formation of covalent

intermolecular cross-links. Collagen cross-linking is crucial for the stability, tensile strength, viscoelasticity and appropriate physiological functions of the collagen fibrils (Eyre *et al.*, 1984; Yamauchi & Mechanic, 1988; Yamauchi, 2002) and can be divided into two types: enzymatic cross-linking, i.e., lysyl oxidase (LOX) mediated cross-links and non-enzymatic cross-linking, e.g., cross-linking induced by sources such as chemical agents, UV light and heat. Several cross-linking agents have been demonstrated to increase the mechanical properties of collagenous tissues and scaffolds (Sung *et al.*, 1999; 1999; Sung *et al.*, 2003; Yao *et al.*, 2005; Castellan *et al.*, 2010). Recently, a natural cross-linking agent, genipin (GE) has been reported to improve the mechanical properties of dentin including ultimate tensile strength and microtensile bond strength (MTBS) between dentin and resin (Bedran-Russo *et al.*, 2007; Bedran-Russo *et al.*, 2008). However, the effects of the GE-induced cross-links on the enzymatic cross-linking and MTBS have not been well documented. In the previous study (chapter 1), by utilizing bovine dentin, we established that 0.5% GE concentration was a sufficient concentration to effectively cross-link dentin collagen. The objectives of this study were to evaluate the effect of 0.5% GE treatment on human dentin collagen and MTBS between composite resin to dentin. We hypothesized that 0.5% GE treatment exerts the effects on human dentin collagen similar to those in bovine dentin and the treatment increases MTBS.

Materials & Methods

Sample Collection and Experimental Treatment for Biochemical Analyses

Extracted intact human molars were used for this study. All samples were stored in distilled water with 0.5% thymol crystals solution at 4°C until use. The pulp, periodontal

ligament and pre-dentin were manually removed, and enamel and cementum were removed using high-speed diamond burs under water cooling. The remaining dentin was pulverized in liquid N_2 by a Spex Freezer Mill (SPEX CertiPrep, Inc., Metuchen, NJ, USA), washed with cold distilled water by repeated centrifugation and lyophilized. In order to obtain sufficient quantities of collagen for biochemical and statistical analyses, two teeth were pooled as a sample and 48 samples (thus, a total of 96 teeth) were generated. After pulverization, the samples were demineralized with 0.5 M ethylenediaminetetraacetic acid (pH 7.4) (EDTA) for 14 days at 4 °C with several changes of EDTA solution, extensively washed with cold distilled water and lyophilized. Approximately two mg aliquots of demineralized dentin (*n*=48) were incubated in 1 mL of either PBS or 0.5% GE for 1 h, 4 h, 12 h and 24 h at 37 °C with agitation. The pH in the solutions was adjusted to 7.4 with 0.5M NaOH. After treatment, the specimens were extensively washed with cold distilled water by centrifugation and lyophilized.

Collagen Stability

Either PBS or 0.5% GE treated-2 mg of demineralized collagen were digested with bacterial collagenase as described in chapter 1. After digestion, the samples were centrifuged for 15 min at 15,000 *g*, the residues (undigested collagen) washed with distilled water and the pooled residues was lyophilized (n=3 in each time point, total sample number=24). The residues were hydrolyzed with 6 *N* HCl (Pierce, Rockford, IL, USA) and subjected to hydroxyproline (Hyp) analysis as described in chapter 1 (Yamauchi & Shiiba, 2008).

Amino acid Analysis and Collagen Cross-link Analysis

Two mg aliquots of dentin collagen treated with PBS or 0.5% GE were reduced with standard NaB^3H_4, hydrolyzed with 300 μL of 6N HCl (Pierce, Rockford, IL, USA) *in vacuo* (see above), after flushing with N_2 gas, for 22 h at 105°C (n=3 in each time point, total sample number=24). The hydrolysates were dried, reconstituted in 300 μl of distilled water, filtered, and then an aliquot of each hydrolysate was subjected to amino acid analysis (see above). The amounts of each amino acid were calculated as residues per 1,000 total amino acids. The hydrolysates with known amounts of Hyp were analyzed for cross-links on a HPLC system as described (Yamauchi & Shiiba, 2008). The reducible immature cross-link, i.e., dehydrodihydroxylysinonorleucine (deH-DHLNL)/its ketoamine was analyzed as the reduced form, i.e. DHLNL. The non-reducible mature cross-link, i.e. pyridinoline (Pyr) was also analyzed simultaneously, and these two cross-links were quantified as moles/mole of collagen. The analyses were done in triplicate in independent experiments.

Sample Collection for MTBS

A total of twenty four sound freshly extracted human molars were selected, cleaned from debris and immediately stored in distilled water with 0.5% thymol crystals solution at 4 C°. The teeth were ground flat and perpendicular to their long axis using 120-grit silicon carbide abrasive paper (Buehler Ltd, Lake Bluff, IL, USA) under running water and mild pressure to expose middle depth dentin. The exposed dentin surfaces were confirmed with a dissecting microscope to ensure that no enamel remained. Dentin surfaces were polished with

wet 240-, 400, and 600-grit silicon carbide abrasive paper (Buehler Ltd, Lake Bluff, IL, USA).

Dentin Surface Pretreatment and Restorative Procedures for MTBS

Specimens were randomly divided according to the dentin treatment and treatment duration): PBS treated (control) groups (treated with PBS for 1 h, 4 h, 12 h and 24 h) and GE treated groups (treated with 0.5% GE in PBS for 1 h, 4 h, 12 h and 24 h). The pH of the solutions was adjusted to 7.4 with NaOH pellets. Prior to treatment, the dentin surface was etched using a 37% phosphoric acid gel (Bisco, Schaumburg, IL, USA) for 15 second (s), then thoroughly rinsed with water for 15 s and kept moist. After etching, the teeth were further divided into four subgroups (*n*= 3 in each subgroup), according to the treatment duration. Teeth were immersed in PBS or 0.5 %GE solution and incubated at 37°C for 1 h, 4 h, 12 h and 24 h. After each time point, specimens were thoroughly rinsed with distilled water for 60 s to remove excess cross-linking agents and buffer, and kept moist. The adhesive system (OptiBond Solo Plus, Kerr, Orange, CA, USA) was used following manufacturer's instructions. A hybrid resin composite restorative material (Z250, 3M ESPE, Saint Paul, Minnesota, USA) was placed over the bonded surfaces incrementally (6 mm total thickness) to allow for gripping during the tensile testing. Increment thickness was limited to 2 mm, and curing was accomplished for 20 s per increment (Ultra-Lume LED5 ULTRADENT INC, South Jordan, UT, USA). Teeth were then stored in distilled water at 37 °C for 24 h. All specimens were sectioned perpendicular to the bonded interface into 0.7 ± 0.2-mm-thick slabs using a slow speed diamond saw (Buehler-Series 15LC Diamond, Lake

Bluff, IL, USA) under cooling water. The specimens were glued on a jig placed on a MTBS

machine (Bisco, Schaumburg, IL, USA) and subjected to tensile force at a crosshead speed of

1 mm min^{-1}. Means and standard deviations were calculated and expressed in MPa.

Statistical Analysis

The statistical evaluations were performed using Stat View software (SAS Institute Inc.,

Cary, NC, USA). Values were expressed as mean +/- standard deviation, and the difference

between the control group and GE groups was compared by two-way ANOVA and Fisher's

PLSD. A p value of less than 0.05 was considered significant.

Results

Collagen Stability

The mean values of Hyp in ~2 mg demineralized dentin matrix in this study was

$1,198.7 \pm 79.8$ nM (n= 3). Hyp analysis showed that while samples in control group for all

time points were almost all digested, in the 0.5% GE, the digestibility markedly decreased in

a time-dependent manner. In 0.5 %GE treatment for 1, 4, 12 and 24 h, 198.5 ± 68.3 nM,

624.6 ± 175.3 nM, 782.0 ± 51.2 nM and 1029.4 ± 127.7 nM of Hyp were recovered,

respectively. Thus, the rate of collagen digestion in the GE treated groups were 80.1% (1 h),

47.9% (4 h), 34.5% (12 h) and 14.1% (24h).

Amino Acid Analysis

Although overall amino acid compositions among the control and GE-treated groups were similar to one another (Table 2.1), as in the case of bovine dentin, significant differences were identified in the values of Hyl and Lys residues. Hyl in 0.5% GE treated for 1 h, 4 h, 12 h and 24 h were significantly decreased in comparison to those of PBS treated groups ($p<0.05$ for 1 h, $p<0.01$ for 4 h, $p<0.0001$ for 12 h and 24 h), and Lys, $p<0.001$ for 4h, $p<0.0001$ for 12h and 24h, respectively. The decreases of Hyl and Lys in 0.5% GE treatment occurred in a time-dependent manner.

Collagen Cross-link Analysis

Two major enzymatic cross-links in dentin collagen, a reducible immature cross-link, DHLNL, and a non-reducible mature cross-link, Pyr, were identified together with other minor cross-links. In addition, unknown reducible compounds that are likely GE-induced reducible cross-links were identified by HPLC analysis in the GE-treated collagen. The quantitative results of the collagen cross-link analyses comparing PBS and 0.5% GE treatment at all time points examined are summarized in Table 2.2. DHLNL in 0.5% GE for 24 h was significant decreased compared with those of controls and 0.5% GE treatment for 1 h and 4 h. ($p< 0.05$). Pyr showed no significant difference between PBS and 0.5% GE treatment groups at all examined time points.

MTBS

The results of MTBS of PBS and 0.5% GE treated groups are summarized in Fig. 2.2. While there was no significant difference in MTBS between PBS and 0.5% GE treated groups for 1 h, those in the GE treated groups for 4 h, 12 h and 24 h were all significantly increased ($p < 0.0001$). The highest bond strength was observed for 0.5% GE treatment for 4 h (58.5 ± 17.7 MPa) which was significantly higher than those of 12 h and 24 h GE treatments ($p < 0.0001$).

Discussion

In the present study, a natural cross-linking agent, GE, was examined to assess its potential effects on human dentin collagen and MTBS. It was found that 0.5%GE treatment for 4 h is sufficient to stabilize dentin collagen matrix and to enhance MTBS.
Although several studies have been performed to assess the effect of GE treatment on collagenous tissues(Touyama *et al.*, 1994; Lee *et al.*, 2003; Fujikawa *et al.*, 1976), the mechanism by which collagen is stabilized by GE is not well understood. As shown in Table 1, only two amino acid residues (Hyl and Lys) were diminished with GE treatment in a time-dependent manner indicating their involvement in GE-induced cross-linking. This is not full agreement with the study conducted by Sung and co-workers (Sung *et al.*, 1998) which reported that Lys, Hyl and arginine residues were involved in GE-induced cross-links. In our study, however, arginine did not change with GE treatment. The difference may be attributed to the treatment duration (~24 h/our study, 3 days/Sung) and/or tissue specific reaction. Normalizing the number of Hyl and Lys residues to 300 residues of Hyp (thus, one collagen molecule) and considering that there are approximately 26 Hyl and 67 Lys residues per

collagen molecule in controls (note: some of the Lys residues are derived from non-collagenous proteins), it is estimated that approximately 31% of the Hyl and 9% of the Lys for 1h, 46% of the Hyl and 40% of the Lys for 4h, 69% of the Hyl and 57% of the Lys for 12h, and 73% of Hyl and 70% of Lys residues of collagen for 24 h were utilized in GE-induced cross-links. The MTBS results of 0.5% GE for 1 h did not significantly alter when compared to that of PBS treatment for 1h and this is consistent with the study conducted by Al-Ammar et al. However, MTBS in 0.5% GE treatment for 4 h, 12 h and 24 h were significantly increased as compared to those treated PBS alone. Interestingly, MTBS slightly but significantly decreased in the samples treated with GE for 12 and 24 h when compared to those treated for 4 h. The reason is not clear at present but one potential explanation would be that when collagen is cross-linked by GE more than certain levels, while collagen stability keeps increasing, the formation of hybrid layer could be compromised. This would lower MTBS. However, the results need to be confirmed and the potential mechanisms should be investigated using a larger sample size combined with careful characterization of the GE-treated hybrid layer.

By cross-link analysis, DHLNL and Pyr, and several unidentified reducible compounds were detected in the GE-treated collagen (data not shown). These unidentified reducible compounds induced by GE increased in a time-dependent manner, thus, they are most likely associated with GE-induced cross-links involving Hyl and Lys residues of collagen. GE treatment for 1 h and 4 h did not significantly alter the enzymatic cross-links, DHLNL and Pyr, when compared to the PBS-treated group (Table 2.2). However DHLNL was significantly decreased at 12 h and 24 h GE treatment when compared to controls. It is possible, therefore, that the GE reacts with not only free Lys and Hyl of collagen but also

with some of the immature cross-link, deH-DHLNL, possibly its aldimine form with a longer period of treatment. The fate of such modifications needs to be pursued in the future studies.

 It is concluded that MTBS increased after dentin collagen was treated with GE for 4 h. Thus this cannot be applied in clinical settings yet. However, it could be possible that the time can be shortened by application methods, e.g. changing temperature (note that all the experiments in this study was performed at 37 °C), pH, or combining with other cross-linking agents. By improving the application time and discoloration, the use of natural collagen cross-linking agents could be a safe and promising approach to improve adhesive restorative procedures and dentin bond strength.

Table 2.1

Amino Acid Composition of GE-Treated Human Dentin

	1h		4h		12h		24h	
	PBS	0.5%GE	PBS	0.5%GE	PBS	0.5%GE	PBS	0.5%GE
Hyp	96.3±5.8	96.6±11.3	100.3±14.3	95.8±6.5	100.8±9.4	90.2±3.9	105.0±15.4	103.6±17.1
Asp	51.9±1.2	52.4±4.5	49.4±3.4	49.6±3.1	48.6±2.3	54.1±5.6	50.2±2.2	53.7±2.6
Thr	17.0±0.5	18.5±0.9	17.4±1.7	16.9±1.5	17.4±1.4	17.8±1.1	17.6±0.5	18.2±1.8
Ser	35.6±1.9	33.4±4.2	35.2±1.4	34.0±1.1	33.1±1.3	36.6±1.9	35.0±2.5	36.5±2.5
Glu	79.9±1.4	80.9±5.3	79.3±2.2	80.9±4.2	78.2±1.3	78.7±5.3	80.1±2.4	83.4±2.7
Pro	123.9±4.4	116.4±6.8	119.1±2.4	123.8±8.4	124.9±6.5	124.1±4.2	122.0±0.3	125.1±5.6
Gly	328.5±10.3	334.1±7.7	321.0±18.1	340.7±8.4	324.9±9.9	346.3±2.0	322.1±14.4	327.8±12.3
Ala	105.7±4.1	111.8±3.3	107.0±2.7	109.3±3.9	108.1±2.6	113.5±3.8	106.4±2.7	109.0±6.4
Val	23.1±0.7	23.5±0.2	22.5±0.5	22.7±2.1	22.8±1.0	22.8±3.4	23.1±1.3	24.0±0.5
Met	2.0±0.3	3.4±2.6	2.3±0.3	1.9±2.2	3.3±1.3	3.1±0.9	2.5±0.5	2.8±1.2
Ile	10.6±0.6	10.7±0.2	9.6±1.1	11.1±1.0	10.1±0.7	10.3±1.3	9.1±0.3	10.8±0.3
Leu	26.9±0.5	27.5±1.0	25.1±2.7	28.0±0.9	25.8±1.4	27.6±1.9	25.4±0.7	28.9±2.6
Tyr	3.6±0.1	2.3±1.4	3.8±0.5	2.3±1.9	3.4±0.4	2.1±1.8	3.0±0.3	3.0±0.2
Phe	11.8±1.2	11.9±0.7	12.9±1.4	13.4±1.0	12.5±1.7	12.9±0.4	11.9±0.4	13.0±1.3
His	5.7±0.2	5.0±0.7	5.8±0.3	4.9±0.6	5.5±0.7	4.4±0.4	5.7±0.1	5.0±0.5
Hyl	8.5±1.2	5.8±1.4[*]	8.5±1.3	4.5±1.3[**]	8.8±1.6	2.3±0.4[****]	8.9±1.3	2.3±0.1[****]
Lys	21.7±1.7	19.7±0.6	23.2±3.8	12.9±0.5[***]	22.9±2.4	8.8±2.5[****]	23.1±4.3	7.0±0.8[****]
Arg	48.2±2.1	44.1±3.5	47.6±6.0	47.1±4.0	49.8±4.6	44.7±2.1	51.0±3.5	48.5±7.8
Total	1000	1000	1000	1000	1000	1000	1000	1000

The average values (n=3) and SDs are shown as relative amounts in 1,000 total residues

*P<0.05 different from the value of PBS (control) for same time point.
**P<0.01 different from the value of PBS (control) for same time point.
***P<0.001 different from the value of PBS (control) for same time point.
P<0.0001 different from the value of PBS (control) for same time point.

Table 2.2

Contents of enzymatic cross-linking of GE-Treated Human Dentin

	1h		4h		12h		24h	
	PBS	0.5%GE	PBS	0.5%GE	PBS	0.5%GE	PBS	0.5%GE
DHLNL	0.691 A ±0.030	0.691 A ±0.038	0.703 A ±0.051	0.706 A ±0.034	0.713 A ±0.026	0.641 A,B ±0.070	0.698 A ±0.025	0.633 B±0.031*
Pyr	0.583 C ±0.003	0.583 C ±0.011	0.598 C ±0.005	0.582 C ±0.012	0.582 C ±0.008	0.560 C ±0.036	0.582 C ±0.042	0.565 C ±0.018

All values in DHLNL and Pyr are expressed in moles/mole collagen (means ± S.D.).
DHLNL; dihydroxylysinonorleucine , Pyr; pyridinoline, n=3
*P< 0.05.
Values with same letters are not statistically significant (p> 0.05).

Figure 2.1

Collagen stability. The mean amounts of digested collagen from 2mg of dentin collagen treated with PBS or three concentrations of 0.5% GE for four treatment durations (1 h, 4 h, 12 h, and 24 h) as showing hydroxyproline residue amounts in supernatant after collagenase digestion for 24 h (n=3).
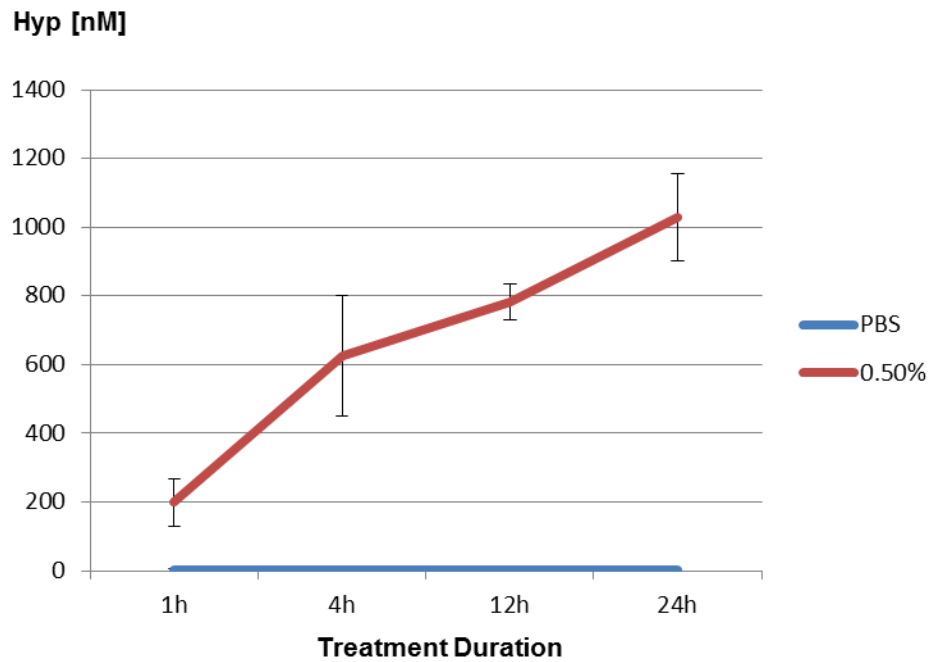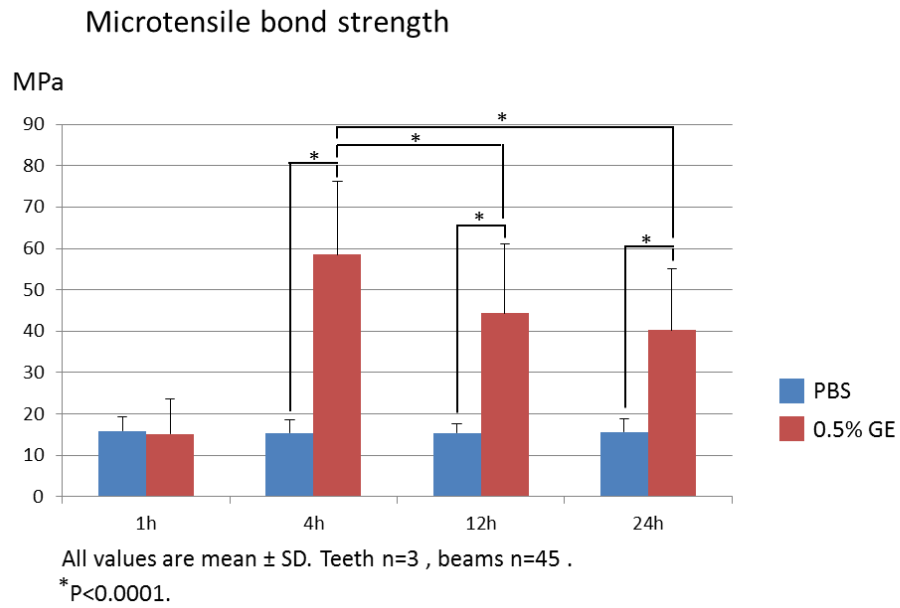
Figure 2.2

MTBS on the samples treated with PBS or 0.5% GE for 1h, 4 h, 12 h and 24 h (tooth n=3,

beams n=45).

Microtensile bond strength

All values are mean ± SD. Teeth n=3 , beams n=45 .
*P<0.0001.

Summary and conclusion

The two studies demonstrate that:

1. The treatment with GE resulted in discoloration and an increase of collagen stability in a dose- and time-dependent manner.

2. The GE-induced cross-links utilize Lys or Hyl residues of collagen and the cross-links increase in a time- and dose-dependent manner

3. Cross-linking induced by 0.5% GE treatment for 4 h is sufficient to improve the mechanical properties of dentin collagen and bond strength.

4. 0.5% GE may modify some of the LOX-mediated immature divalent cross-links when treated for a longer period of time.

5. GE-induced cross-linking for a long period of time (12 and 24 h) may result in slight decreases in MTBS.

Thus, while it requires more studies and modifications, GE treatment could be an efficient approach to improve mechanical properties of dentin collagen matrix and the longevity of resin-dentin bond.

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