DENTAL MATERIALS 30 (2014) 227–233

Available online at www.sciencedirect.com

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Stabilization of dentin matrix after cross-linking treatments, in vitro

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ARTICLE INFO

Article history:
Received 26 November 2012
Received in revised form
19 June 2013
Accepted 20 November 2013

Keywords:
MMPs
Collagen
Dentin
Cross-linkers
Glutaraldehyde
EDC

ABSTRACT

Objectives. To evaluate the effect of EDC on elastic modulus (E), MMPs activity, hydroxyproline (HYP) release and thermal denaturation temperature of demineralized dentin collagen.

Methods. Dentin beams were obtained from human molars and completely demineralized in 10 wt% H_3PO_4 for 18 h. The initial E and MMP activity were determined with three-point bending and microcolorimetric assay, respectively. Extra demineralized beams were dehydrated and the initial dry mass (DM) was determined. All the beams were distributed into groups ($n = 10$) and treated for 30 s or 60 s with: water, 0.5 M, 1 M or 2 M EDC or 10% glutaraldehyde (GA). After treatment, the new E and MMP activity were redetermined. The beams submitted to DM measurements were storage for 1 week in artificial saliva, after that the mass loss and HYP release were evaluated. The collagen thermal denaturation temperature (TDT) was determined by DSC analysis. Data for E, MMP activity and HYP release were submitted to Wilcoxon and Kruskal–Wallis or Mann–Whitney tests. Mass loss and TDT data were submitted to ANOVA and Tukey tests at the 5% of significance.

Results. EDC was able to significantly increase collagen stiffness in 60 s. 10% GA groups obtained the highest E values after both 30 and 60 s. All cross-linking agents decreased MMP activity and HYP release and increased TDT temperature. Significant differences were identified among EDC groups after 30 or 60 s of cross-linking, 1 M or 2 M EDC showed the lowest MMP activity.

Significance. Cross-linking agents are capable of preventing dentin collagen degradation. EDC treatment may be clinically useful to increase resin-dentin stability.

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http://dx.doi.org/10.1016/j.dental.2013.11.007

1. Introduction

Contrary to stable resin-enamel bonds, effective, long-lasting dentin bonds remains a challenge to clinicians [1]. The hybrid layer is considered the main structure responsible for micromechanical retention of resin restorations and also responsible for sealing the dentin [2]. However, this important structure is the most vulnerable area of the adhesive interface [3–5].

Bond degradation has been attributed to hydrolytic breakdown of resin adhesive or dentin collagen, or both. TEM examination of the hybrid layers shows replacement of collagen fibrils by water [6]. This degradation is thought to be due to endogenous MMPs and cathepsins in acid-etched dentin. Demineralized dentin contains matrix-bound metalloproteinases-2, -3, -8, -9 and -20 (MMPs) and cathepsins [7,8] in their active forms. These enzymes are exposed and activated by acid-etching and can slowly degrade collagen fibrils [9–12] within the hybrid layer, resulting in a significant loss of bond strength of 36–70% within 12–14 months [13,14].

Cross-linking agents are capable of non-specifically cross-linking protein such as collagen and dentin proteases [15,16]. 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) is a stable isomer of carbodiimide, capable of cross-linking proteins by activating the carboxyl group of glutamic and aspartic acids and then reacting with ε-amino groups present in protein molecules, resulting in the creation of covalent cross-links [17,18]. Cross-linking increases the mechanical properties of dentin collagen and makes the fibrils more resistant to degradation [15]. Furthermore, EDC has no transdentinal cytotoxic effect (Scheffel et al., unpublished data). However, previous reports of EDC treatment of dentin used 10 min to 4 yrs that are not clinically relevant [15].

Matrix-bound dentin proteases contain numerous residues of glutamic and aspartic acids in their structures. Thus, if EDC reacts with demineralized dentin collagen [15], it should also react with any proteases bound to collagen. Such protease cross-linking should reduce the molecular mobility of dentin MMPs and cathepsins, thereby inactivating them [19]. Additionally, besides bonding between collagen polypeptide chains, it is plausible that EDC could cross-link the catalytic sites of dentin proteases, thereby blocking resin-dentin bond degradation. The purpose of this study was to evaluate the effect of 30 or 60 s EDC application on elastic modulus, total matrix-bound MMPs activity, hydroxyproline release and thermal denaturation temperature of completely demineralized dentin. The null hypotheses were that cross-linker-treated and untreated dentin do not differ regarding elastic modulus, total MMP activity, amount of hydroxyproline release and thermal denaturation temperature, and the time of application does not influence these properties.

2. Materials and methods

Fifty extracted human third molars were obtained from 18 to 21 year-old patients with informed consent under a protocol approved by the Georgia Regents University. The teeth were stored frozen until required. After thawing, the enamel and superficial dentin were removed using an Isomet saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. One 1 mm-thick dentin disk was produced from the mid-coronal dentin of each tooth.

2.1. Elastic modulus

One-hundred dentin beams (1 mm × 1 mm × 3 mm) were sectioned from the dentin disks. The beams were completely demineralized in 10 wt% H_3PO_4 (pH 1) for 18 h at 25 °C and rinsed with deionized water for 2 h at 3–4 °C. The initial elastic modulus of each demineralized beam was determined by three-point flexure [20]. An aluminum testing jig with a 2.5 mm span between supports was fixed to the bottom of a glass Petri dish. Specimens were tested under compression, while immersed in distilled water, by means of a testing machine (Vitrodyne V1000, Liveco Inc., Burlington, VT, USA), with a 100 g load cell, at a crosshead speed of 1 mm/min. After maximum displacement, it was returned immediately to 0% stress to prevent creep of the demineralized collagen. Load–displacement curves were converted to stress–strain curves, and the apparent modulus of elasticity was calculated at 15% strain. Then the beams were randomly divided into 10 groups ($n = 10$), so that the mean initial elastic modulus of each group was statistically similar. To calculate elastic modulus of each specimen, the steepest slope of the linear portion of the stress–strain curve was placed in the following formula:

$$E = \frac{mL^3}{4bd^3}$$

m = slope (N/mm); L = support span (mm); d = thickness of beam (mm); b = width of beam (mm). Because specimen displacement was estimate from cross-head displacement, and the specimens thickness was not one-sixteenth of the length [20] the calculated elastic moduli are approximate. Although both the two supports and the third mid-beam compressive member may have slightly deformed the surface of the specimens, that deformation was the same before and after treatment. We were more interested in changes in modulus of elasticity, rather than their absolute values.

2.2. Pre-treatment MMP activity of dentin

To determine the initial total MMP activity, each beam was placed into 200 μl of a generic MMP substrate (Sensolyte Generic MMP colorimetric assay kit – catalog No. 72095, AnaSpec Inc. Fremont, CA, USA) for 60 min at 25 °C in a 96-well plate. At the end of 60 min, the total MMP activity was determined by measuring the absorbance of the wells at 412 nm in a plate reader (Synergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) against blanks. The substrate is cleaved by MMPs 2, 8 and 9 in dentin and releases a sulfhydryl group that reacts with Ellman's Reagent (5,5′-dithiobis(2-nitrobenzoic acid). The final product of this reaction, 2-nitro-5-thiobenzoic acid (TNB), turns the medium yellow and can be read by a plate reader. All chemicals were purchased from Sigma/Aldrich Chemical Co and used as received.