DENTAL MATERIALS XXX (2015) XXX–XXX

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/dema

Occlusal loading and cross-linking effects on dentin collagen degradation in physiological conditions

Gianluca Turco[a], *Andrea Frassetto*[a], *Luca Fontanive*[a], *Annalisa Mazzoni*[c], *Milena Cadenaro*[a], *Roberto Di Lenarda*[a], *Franklin R. Tay*[b], *David H. Pashley*[b], *Lorenzo Breschi*[c,*]

[a] *Department of Medical Sciences, University of Trieste, Piazza dell'Ospitale 1, 34125 Trieste, Italy*
[b] *Department of Oral Biology, Georgia Regents University, College of Dental Medicine, Augusta, GA, USA*
[c] *Department of Biomedical and Neuromotor Sciences, DIBINEM, University of Bologna, Italy*

ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form
18 October 2015
Accepted 30 November 2015
Available online xxx

Keywords:
Matrix metalloproteinases (MMPs)
Collagen(s)
Demineralization
Dentin
Cross-linking agent
Mastication Stress

ABSTRACT

Objective. This study evaluated the ability of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) to improve the stability of demineralized dentin collagen matrices when subjected to mechanical cycling by means of Chewing Simulation (CS).

Methods. Demineralized dentin disks were randomly assigned to four groups ($N = 4$): (1) immersion in artificial saliva at 37 °C for 30 days; (2) pre-treatment with 0.5 M EDC for 60 s, then stored as in Group 1; (3) CS challenge (50 N occlusal load, 30 s occlusal time plus 30 s with no load, for 30 days); (4) pre-treatment with 0.5 M EDC as in Group 2 and CS challenge as in Group 3. Collagen degradation was evaluated by sampling storage media for ICTP and CTX telopeptides.

Results. EDC treated specimens showed no significant telopeptides release, irrespective of the aging method. Cyclic stressing of EDC-untreated specimens caused significantly higher ICTP release at day 1, compared to static storage, while by days 3 and 4, the ICTP release in the cyclic group fell significantly below the static group, and then remained undetectable from 5 to 30 days. CTX release in the cyclic groups, on EDC-untreated control specimens was always lower than in the static group in days 1–4, and then fell to undetectable for 30 days.

Significance. This study showed that chewing stresses applied to control untreated demineralized dentin increased degradation of collagen in terms of CTX release, while collagen crosslinking agents may prevent dentin collagen degradation, irrespective of simulated occlusal function.

* Corresponding author at: Dental School, Department of Biomedical and Neuromotor Sciences, DIBINEM University of Bologna, Alma Mater Studiorum Via San Vitale, 59 40125 Bologna, Italy. Tel.: +39 051 20 88139; fax: +39 051 22 5208.
E-mail address: lorenzo.breschi@unibo.it (L. Breschi).

http://dx.doi.org/10.1016/j.dental.2015.11.026
0109-5641/

1. Introduction

In order to bond adhesive resins to dentin, dentists acid-etch dentin surfaces to remove the mineral phase, then solvated comonomer mixtures are infiltrated into the completely demineralized matrix to form the hybrid layer. Unfortunately, the bottom of the hybrid layer is not well infiltrated by resin and therefore is saturated with water, leaving completely demineralized dentin matrix, with stiffness values of only 10 MPa, to undergo excessive stresses and strain during mastication [1]. According to Singh *et al.* [2], poorly infiltrated hybrid layers show the highest stress concentrations, which combined with the absence of the mineral matrix render the collagen matrix more vulnerable to degradation by means of endogenous matrix-metalloproteinases (MMPs) [3].

The dentin organic matrix has a specific nanostructural organization characterized by a complex network of type I collagen fibrils representing approximately 90 wt.% of the dentin organic phase [4]. Type I collagen has been widely investigated over the past two decades and its stability is believed to be a crucial factor for the durability of the hybrid layer and the effectiveness of the resin–dentin bond [5–7].

During etch-and-rinse adhesive procedures, the collagen matrix is exposed by acid-etching. MMPs are inactive in mineralized dentin and bone [5,6], but may be activated during acid-etching and, slowly hydrolyze sub-optimally infiltrated collagen fibrils within the hybrid layer [3]. This degradation results in the loss of resin–dentin bond strength and premature failure of the restoration [8–11]. In addition to MMPs, cysteine cathepsins (in particular cathepsin K) are also present in mineralized dentin [3,9,12], which may be responsible for collagen degradation within hybrid layers. The enzymatic activity of both MMPs and cathepsins can be assayed by quantifying the release of telopeptides derived from the demineralized dentin collagen matrix [13–15].

Collagen fibrils in hybrid layers can be cross-linked and rendered more resistant to collagenolysis [16]. In particular, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), an imide-based zero-length cross-linking agent, has been shown to be effective for cross-linking dentin collagen thereby inhibiting enzymatic degradation of denuded collagen fibrils within the hybrid layer and thereby improving bond durability over time [16–18].

Research protocols investigating *in vitro* aging of the hybrid layer should consider all possible parameters involved in the degradation of collagen (*i.e.* thermal, mechanical, physical and chemical factors) to obtain clinically reliable results. In particular, simulation of mastication cycles (*i.e.* chewing simulation, CS) is paramount for replicating the dynamic physiological conditions of human mastication *in vitro* [19–21]. Previous studies suggested that host-derived MMPs and cathepsins act synergistically to degrade the dentin collagen network [22]. Recently published work reports that load cycling decreased collagen degradation when PA or EDTA-demineralized dentin were loaded for 13.8 h under 3-point flexure at 49 N loading [23]. In that study, the authors only loaded specimens for 13 h over a 30-day experiment.

Thus, the purpose of the present study was to evaluate the ability of EDC pre-treatment to improve the stability of demineralized dentin collagen matrices when those matrices were subjected to mechanical cycling in physiological conditions (pH 7.4), by quantifying the release of telopeptide fragments over time. The null hypotheses tested were that (1) prolonged chewing simulation has no effect on collagen degradation over time, and that (2) pretreatment of demineralized dentin with EDC has no protective effect on cyclically-loaded dentin over time.

2. Materials and methods

2.1. Specimen preparation

Sixteen extracted noncarious human molars were collected after obtaining patients' informed consent for using their extracted teeth for research purposes, under a protocol approved by the institutional Review Board of the University of Trieste (Italy). The teeth were stored at 4 °C in 0.5% chloramine-T solution for no more than 1 month before use. Enamel, cementum, and pulpal soft tissues were completely removed from each tooth. A dentin slab (1.0 ± 0.1 mm thick) was obtained from the mid-coronal portion of each tooth using a slow-speed diamond saw (Isomet 5000, Buehler Ltd., Lake Bluff, IL, USA) under continuous water-cooling.

The dentin slabs were completely demineralized in 10 wt% phosphoric acid (pH 1) at 25 °C for 24 h. Demineralized dentin slabs were thoroughly rinsed in deionized water under constant stirring at 4 °C for 72 h [24]. Collagen slabs were then cut into circular disks (6.0 ± 0.2 mm in diameter and 1 mm thick) by means of a surgical biopsy punch (Kai Europe GmbH, Solingen, Germany). The collagen disks were then randomly assigned to four treatment groups ($N = 4$) with different storage conditions.

Group 1 (Static): each specimen was immersed in a centrifuge tube containing 0.5 mL of artificial saliva (KCl 12.92 mM, KSCN 1.95 mM, $Na_2SO_4 \cdot 10H_2O$ 2.37 mM, NH_4Cl 3.33 mM, $CaCl_2 \cdot 2H_2O$ 1.55 mM, $NaHCO_3$ 7.51 mM, $ZnCl_2$ 0.02 mM, HEPES 5 mM, pH 7.4) and stored at 37 °C for 30 days without shaking.

Group 2 (EDC + Static): specimens were pre-treated with 0.5 M EDC solution (pH 6.3) for 60 s, rinsed with distilled water for 10 min, and then stored in the same manner as Group 1.

Group 3 (Chewing Simulation, CS): specimens were challenged with cycling loading to simulate occlusal function. For this purpose, 1 mm thick specimens were placed at the bottom of a chewing simulator (CS-4.4, SD Mechatronik GmbH, Munich, Germany) sealed chamber (Fig. 1), covered with 0.5 mL artificial saliva and compressed to a thickness of 0.50 ± 0.01 mm using a 50 N occlusal load. The simulated masticatory load was applied for 30 s, followed by 30 s period for specimen recoil in which specimens were left unloaded, before the application of a new occlusal cycle. The mastication cycle was repeated for 30 days at a temperature of 37 °C (approximately 43,200 cycles *in toto*). Cyclic loading was done for 43,200 min or 30 days.

Group 4 (EDC + CS): specimens were treated with 0.5 M EDC solution for 60 s, rinsed with distilled water for 10 min, and then challenged with CS as in Group 3.

Please cite this article in press as: Turco G, et al. Occlusal loading and cross-linking effects on dentin collagen degradation in physiological conditions. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.11.026