

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

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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 (50N occlusal load, 30s 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.

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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, Na₂SO₄·10H₂O 2.37 mM, NH₄Cl 3.33 mM, CaCl₂·2H₂O 1.55 mM, NaHCO₃ 7.51 mM, ZnCl₂ 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.

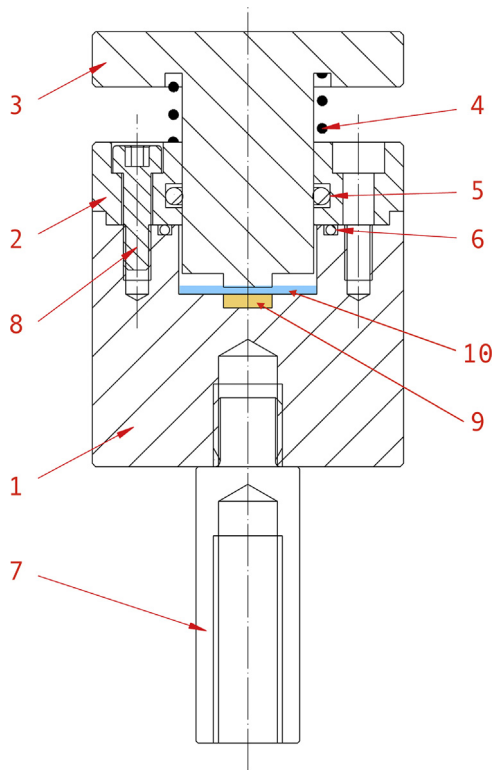


Fig. 1 – Schematic representation of the Chewing Simulator sealed chamber used to challenge the collagen specimens over a 30-day period. (1) Chamber, (2) Lid, (3) Piston, (4) Spring, (5) O-Ring, (6) O-Ring, (7) Pivot, (8) Screw, (9) Dentin specimen, (10) Medium.

For all the specimens, the artificial saliva was changed every 24 h and the aging medium was collected and frozen at -20°C .

2.2. Assays for CTX and ICTP

MMP-induced collagen degradation was assessed using the ICTP ELISA kit (UniQ ICTP EIA; Orion Diagnostica, Espoo, Finland) for quantification of solubilized type I collagen C-terminal cross-linked telopeptide (ICTP) fragments released during the 30-day aging period (Static or CS). This assay has a measurement range from 1 to $50\ \mu\text{g/L} \pm 0.3\ \mu\text{g/L}$ of medium for ICTP. Briefly, the aging medium was diluted 1:10 in saline and pipetted ($50\ \mu\text{L}$ per well) in quadruplicate into a 96-well plate. Procedures were then performed according to the manufacturer's instructions. Absorbance was measured at 450 nm using a plate reader (GloMax Multi Detection System, Promega Corp., Madison, WI, USA).

Cathepsin K degradative activity was measured by quantifying the amount of solubilized C-terminal peptide (CTX) in the aging medium over the 30-day aging period, using the Serum CrossLaps ELISA kit (Urine BETA CrossLaps ELISA, Immunodiagnostic Systems, Boldon, UK). This assay has a measurement range from 0.20 to $40\ \mu\text{g/L} \pm 0.8\ \mu\text{g/L}$ of medium for CTX. Briefly, specimens containing the aging medium or the controls were diluted 1:4 in "standard 0" provided by the kit and pipetted ($50\ \mu\text{L}$ per well) in quadruplicate in a 96-well

plate. Procedures were then performed according to the manufacturer's instructions. Absorbance was measured at 450 nm using the plate reader. Reference absorbance was measured at 650 nm and then subtracted from the measurement determined at 450 nm.

2.3. Statistical analysis

The ICTP and CTX release rates were analyzed separately. For each telopeptide, data from all groups (in ng telopeptide/mg demineralized dry dentin) were assessed to determine if their normality (Kolmogorov–Smirnov test) and equal variance (Levine test) assumptions were violated. Since the normality and equality variance assumptions of the data were valid, they were analyzed by two-way analyses of variance (one for ICTP and the other for CTX), with dentin treatment (EDC or NO EDC) and aging conditions (Static or CS) as factors. Post-hoc multiple comparisons were performed with the Tukey test using SPSS Statistics 21 (IBM SPSS Statistics; SPSS Inc., Chicago, IL, USA). Statistical significance was pre-set at $\alpha=0.05$.

3. Results

The means and standard deviations of ICTP and CTX fragments detected in the storage medium are reported respectively in Figs. 2 and 3.

Dentin pretreatment with 0.5M EDC resulted in minimal release of ICTP or CTX telopeptide fragments from specimens stored either in static conditions (Group 2) or cyclically-stressed (Group 4), which was below the detection level of the assay throughout the entire 30-day period (Figs. 2 and 3).

If EDC pretreatment was not performed, release of ICTP fragments from was related to aging time (Fig. 2), with the highest amount of ICTP released at day 1 irrespective from the aging condition (static i.e. Group 1 = $21.9 \pm 3.1\ \text{ng ICTP/mg}$ demineralized dry dentin; CS i.e. Group 3 = $33.0 \pm 1.5\ \text{ng ICTP/mg}$ demineralized dry dentin), which decreased significantly over time ($p < 0.05$), being below detection level by the assay ($1\ \text{ng ICTP/mg}$ demineralized dry dentin) after day 4.

The comparative analysis of the first 4 days of aging between the groups without EDC pretreatment showed that at day 1, ICTP release in the CS group (Group 3) was higher compared to static group (Group 1; $p < 0.05$; Fig. 2), while no significant difference was found between the two groups (Group 1 vs Group 3; $p > 0.05$) at day 2, and at days 3 and 4 the ICTP release was higher in static conditions compared to CS ($p < 0.05$). Due to this conflicting trend, ICTP total release of Group 1 vs Group 3 showed no significant difference after the 30-day aging period (Group 1 = $43.3 \pm 7.9\ \text{ng ICTP/mg}$ demineralized dry dentin vs Group 3 = $47.4 \pm 5.8\ \text{ng ICTP/mg}$ demineralized dry dentin; $p > 0.05$).

The release of CTX fragments (Fig. 3) of control specimens untreated with EDC showed similar decreasing release behavior as a function of time (even if CTX fragment release was at least one order of magnitude lower than ICTP release) being below detection level by the assay ($0.1\ \text{ng CTX/mg}$ demineralized dry dentin) from day 5 to day 30, irrespective from static or CS aging.

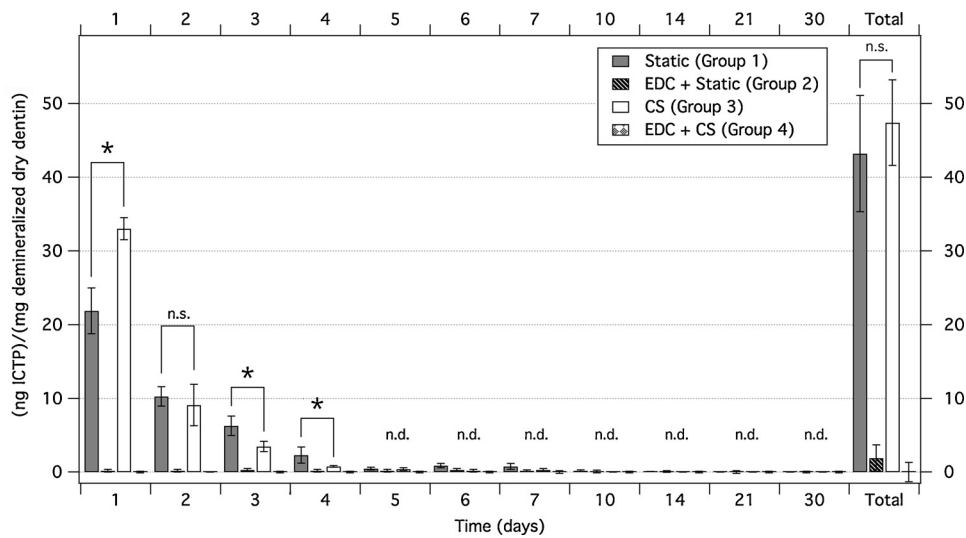


Fig. 2 – Means ± standard deviation of C-terminal telopeptide ICTP release from phosphoric acid-demineralized dentin disks. Values are expressed as ng ICTP/mg demineralized dry dentin. Bars identified by asterisk are significantly different ($p < 0.05$), while n.s. indicates no significant difference ($p > 0.05$); n.d. indicates non-detectable amounts (lower than 1 ng ICTP/mg demineralized dry dentin).

When static and CS groups were compared, the release of CTX fragments of Group 3 specimens (CS without EDC pretreatment) showed significantly lower CTX telopeptide release from day 1 to day 4 ($p < 0.05$) compared with Group 1 specimens (static aging condition without EDC pre-treatment). Similarly, the total amount of CTX telopeptide fragments released over the 30-day period (Fig. 3) was higher in specimens aged under static conditions than specimens aged with CS (Group 1 = 3.4 ± 1.3 ng CTX/mg demineralized dry dentin vs Group 3 = 1.3 ± 0.3 ng CTX/mg demineralized dry dentin; $p < 0.05$).

There were no significant interactions between pretreatment (EDC or NO EDC) and storage conditions (Static or CS) ($p = 0.451$).

4. Discussion

The results of the present study showed that EDC pretreatment significantly reduced the release of both ICTP and CTX telopeptide fragments in demineralized dentin collagen matrices that were aged under both static and dynamic

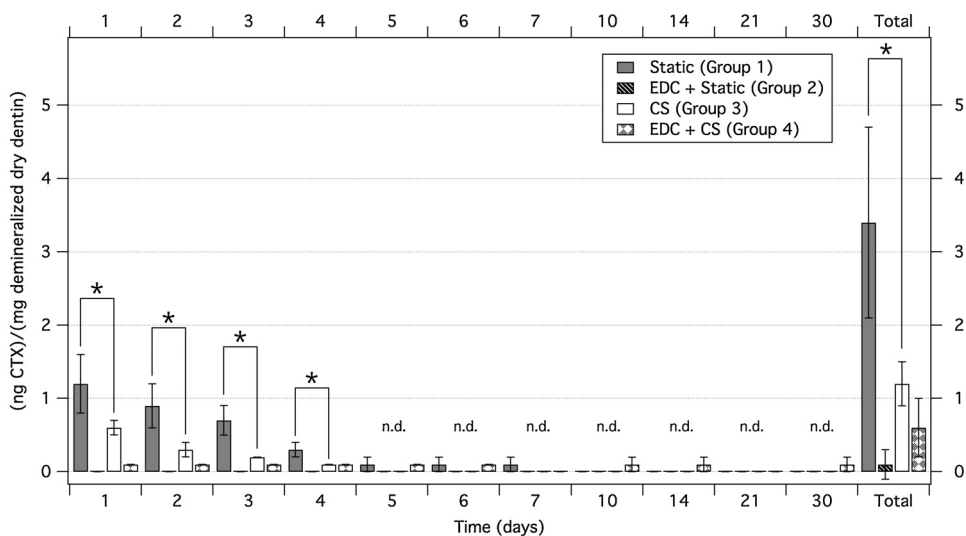


Fig. 3 – Means ± standard deviation of C-terminal telopeptide CTX release from phosphoric acid-demineralized dentin disks. Values are expressed as ng CTX/mg demineralized dry dentin. Bars identified by asterisk are significantly different ($p < 0.05$); n.s. indicates no significant difference ($p > 0.05$); n.d. indicates non-detectable amounts (lower than 0.1 ng CTX/mg demineralized dry dentin).

conditions at physiological pH. Thus, the first null hypothesis tested that EDC pre-treatment has no effect on the release of telopeptide fragments from demineralized dentin has to be rejected.

The second null hypothesis that chewing simulation has no effect on collagen degradation over time has to be partially rejected, since differences were found only for control specimens that were not-treated with EDC.

Indeed the ICTP and CTX release in specimens that were not pretreated with EDC followed an interesting trend. At day 1, ICTP release in the CS group (Group 3) was higher compared to static group (Group 1; $p < 0.05$; Fig. 2), while no significant difference was found at day 2 (Group 1 vs Group 3; $p > 0.05$), and at days 3 and 4 the ICTP release was higher in static conditions compared to CS ($p < 0.05$). Due to this trend, ICTP total release of Group 1 and Group 3 showed no significant difference ($p > 0.05$; Fig. 2). On the contrary, CTX fragments showed always higher release for EDC-untreated specimens stored in static conditions (Group 1) compared to CS (Group 3) for the first four days (Fig. 3; $p < 0.05$), resulting in a higher total release at the end of the experiments for Group 1 compared to Group 3 (Fig. 3). We speculate that these results were due to the size-exclusion phenomena reported by Toroian et al. [25] in which large molecules, as ICTP, are hampered from diffusion through the collagen matrix, while smaller molecules like CTX can easily flow throughout demineralized specimen. When the collagen matrix underwent chewing simulation with cyclic loading, the compressive deformation altered the three-dimensional structure of collagen decreasing the possibility of diffusion of CTX fragments compared to specimens stored under static conditions.

The mechanisms in which type I collagen molecules are enzymatically cleaved within their C-terminal region into distinct telopeptide fragments (i.e. ICTP and CTX) was previously highlighted and correlated to MMPs and cathepsin K activity, respectively [13]. Recently, this quantitative technique has been adopted for evaluating collagen degradation in acid-etched dentin [14,15]. Using this technique in the present study, we observed that EDC pretreatment clearly affect collagen degradation at least for 4 days. The role of the storage conditions on the collagen degradation is still to be clarified; our group is planning a new set of experiments to elucidate this aspect.

Cross-linking agents such as EDC, riboflavin, genipin, proanthocyanidin or glutaraldehyde have been used experimentally to increase the mechanical and structural stability of dentin collagen and to create a durable and more stable hybrid layer [16,17,26]. The zero-length cross-linking agent, EDC, contains a functional group with the formula $RN=C=NR$. It reacts with ionized carboxyl groups in proteins to form an O-acylisourea intermediate that reacts with a non-proteinated amino group and a protein chain to form a stable covalent amide bond between two proteins with the only product being urea [27]. Carbodiimide is considered one of the least cytotoxic cross-linking agent, and its cross-links are very stable [28,29].

There is ample evidence to support dentin collagen reinforcement and strengthening through EDC cross-linking to improve the bond strength and structural integrity of the resin/dentin interface over time. Indeed, EDC can reduce enzymatic and hydrolytic degradation over time through the

formation of inter- and intra-molecular crosslinks [17,18,30]. In addition, EDC may inactivate exposed MMPs bound to matrix collagen, possibly by altering the 3-D structure of their catalytic or allosteric domains [18].

To explain MMP inactivation by cross-linking agents, the mechanism that has been proposed is based on 3D conformational changes in the enzyme structure that may be achieved via irreversible changes induced within the catalytic domain or allosteric inhibition of other modular domains, which co-participate in collagen degradation [9,26,31]. This cross-linking ability inactivates the active sites of dentin proteases through the reduction of the molecular mobility of the active site or by changing negatively-charged ionized carboxyl groups into positively charged amides. Furthermore, EDC can cross-link both the helical and especially telopeptide domains in collagen and may also prevent telopeptidase activity that would normally remove bulky telopeptides from the specific peptide bond of collagenases [27]. Increase in collagen stiffness may prevent MMPs from “unwinding” collagen peptides [32]. Since this “unwinding” is necessary to allow the collagenolytic MMPs catalytic site to cleave the specific Gly-Ileu peptide bonds [3,9], it would also effectively inhibit MMPs functional activity. In dentin matrices, the endogenous proteases of dentin (MMPs-2, -8, -9, -20 and cathepsins B and K) are thought to bind to collagen molecules as they aggregate extracellularly. Once collagen fibrils aggregate, most of the proteases are considered to be tightly bound to collagen (MMPs) or proteoglycans (cathepsin K).

When Toroian et al. [25] developed their gel exclusion chromatography method for measure the size-exclusion characteristics of type I bone collagen, they concluded that molecules smaller than 6 kDa can diffuse in and out of collagen water but molecules larger than 40 kDa are excluded. A logical extension of their results is that molecules larger than 40 kDa cannot diffuse out of collagen fibril aggregates. Pro- and active forms of MMP-2 and MMP-9 range from 72 kDa (pro) to 62 kDa (active) and 92 kDa (pro) to 67 kDa (active), respectively, and therefore would have trouble penetrating into collagen and also diffusing out of collagen macromolecules [33].

MMP-2 and -9, which are not collagenases *per se* but are gelatinases and telopeptidases, were found to be present in dentin [3]. When MMP-2 or -9 attacks C-terminal telopeptides on exposed free matrix surfaces, some of the cleaved ICTP fragments can diffuse into the incubation medium and be detected by ELISA assays.

Deeper collagen molecules that are completely surrounded by neighboring collagen molecules may be cleaved by bound MMPs and cathepsin K, but those telopeptides may be trapped within the depths of the matrix. Although C-terminal telopeptides like ICTP are only 10–20 kDa [34], we have found size-exclusion results in demineralized dentin using 10 kDa tracers [35]. Thus, cleaved surface telopeptides may diffuse into the incubation medium, while cleaved telopeptide fragments far below the exposed surface may not diffuse out of collagen matrices. This may explain the time-dependent decrease in the release of telopeptides. The surface telopeptide fragments may become depleted in 3–5 days.

Nagase et al. [36] review shows that the collagen binding segment of MMP-2 is very close to the active site. Thus, when mineralized dentin is demineralized with phosphoric

acid, MMPs are uncovered and activated. Presumably, they are bound to collagen by their fibronectin and hemopexin binding sites, while their nearby active sites may attack the very collagen to which they are bound. Presumably, bound MMPs have some freedom of movement, but not much. One would expect bound but activated MMPs to attack helical collagen (MMP-8 in dentin) or C-terminal telopeptides (MMPs-2 and -9). Over time (days), the substrates for these MMPs (*i.e.* insoluble collagen) would slowly solubilize and diffuse from the residual insoluble collagen. If the MMPs remain bound to the same part of the matrix would not the enzyme run out of substrate?

Because dentin collagen does not turn over, it is the most cross-linked collagen in the body. Indeed, these cross-links transmit mechanical force throughout the matrix [37]. When true collagenases cleave collagen molecules into 1/4–3/4 fragments, both types of fragments remain covalently cross-linked to adjacent collagen molecules. This is part of the size-exclusion meshwork in bone and dentin collagen. Collagen molecules are physically oriented in collagen fibrils slightly diagonally [13,37]. This brings the more globular C-terminal end of collagen molecules to the surface in a position that sterically blocks access of MMP-8 (or other true collagenases) to the Gly-Ile peptide bond on α -2 chains. When telopeptidases (MMP-2 and -9) attack the C-terminal telopeptides, they remove steric restrictions to true collagenases. True collagenases then can bind to collagen and unwind the triple helix, allowing access to the critical peptide bond. Thus, telopeptidase activity is very important. Garnero *et al.* [13] reported that ICTP telopeptides are only released by the action of MMPs, and that CTX fragments are only released by cathepsin K.

Why did the release of ICTP and CTX fall over the first four days of the experiment? Perhaps the C-terminal fragments that were measured came from the exposed surfaces that were less cross-linked to adjacent collagen peptides than were deeper telopeptides surrounded by cross-links. That is, deeper telopeptides may have been cleaved but remained cross-linked and could not solubilize. Alternatively, perhaps MMPs bound to collagen, depleted accessible telopeptides from within their “zone of molecular mobility” and they ran out of accessible substrate.

We purposely selected our compressive strain to produce –50% strain that was held for 30 s. It was hoped that such compression would bring un-hydrolyzed telopeptides within “reach” of the bound MMPs. Clearly, that strategy was unsuccessful.

When completely demineralized matrices are placed under –50% strain, the aqueous media is literally squeezed out of the matrix as the fluid-filled interfibrillar spaces are compressed upon themselves during the 30 s compressive stage. In the current study, that compressive strain was maintained for 43,200 cycles for 1 min or 43,200 min or 30 days, that was much longer than the 13 h of loading used by Toledano *et al.* [23]. The endogenous proteases of the dentin matrix are hydrolases. That is, they add water across specific peptide bonds to cleave collagen peptides. If cyclic loading temporarily “dehydrates” the matrix, the enzymes may have less time and less water to hydrolyze the peptides. If the enzymes require a certain molecular mobility during this process, that mobility may be severely restricted during loading. In the Toledano *et al.* [23] study, the authors used compressive cyclic loading. In this

way, the top surface of the beam was exposed to compressive stresses that would express free water from interfibrillar channels between collagen fibrils. Those stresses may also temporarily remove free water from the intrafibrillar compartment of collagen fibrils. As free water is squeezed from the matrix, the water may induce fluid shear stress on matrix molecules that may denature endogenous proteases. Future experiments should vary the rate of loading 10- or 100-fold to vary fluid shear stress to determine if that alters the rate of release of ICTP or CTX.

Thus, the results of both the current study, and the Toledano *et al.* [23] study are mutually supportive. Toledano *et al.* [23] described the results as showing that cyclic loading “enhanced the resistance of demineralized dentin matrices to degradation by endogenous dentin proteases.”

We would describe the effect of cyclic loading as causing alterations of matrices, rather than “enhancing collagen’s resistance to enzymatic degradation”, as Toledano *et al.* [23] described their effect of cyclic loading on ICTP release.

The above speculations need to be confirmed by careful future experimentation. They provide a testable rationale for the observation that cyclic loading decreases degradation of dentin collagen matrices.

In the current study, we sampled both ICTP and CTX telopeptides in pH 7.4 media. These conditions are recognized to be the optimum pH for MMPs whereas cathepsins are more active in acidic solutions (*i.e.* pH 5.0–5.5) [13,38]. Previous studies showed that in physiological conditions cathepsins still remain active, albeit at a lower level [22,39]. Kometani *et al.* [39] reported that human cathepsin K activity expressed 91% of its maximal activity at pH 5.5, 85% of its maximal activity at pH 6.5 and 11% of its maximal activity at pH 7.5. We repeated those experiments and found that cathepsin K activity in demineralized dentin matrices at pH 7.4 was 11.3% of its activity at pH 5.5 (data not shown). Present study confirmed that physiological conditions, *i.e.* pH 7.4, the usual pH for hybrid layers, exhibited CTX levels which are at least one-tenth of the ICTP ones. This validates the method of analyzing incubation media for both ICTP and CTX to simultaneously follow MMPs and cathepsin K activity in dentin matrices.

Although Toledano *et al.* [23] reported that cyclic loading of mineralized and phosphoric acid or EDTA demineralized dentin beams decreased collagen degradation after 4 weeks of storage, they only used compression of beams under 49 N loading. Those authors did not report the degree of strain in those studies. Their specimens were submitted to 100,000 cycles at 2 Hz for 13 h at out of 30 days. In our study, specimens subjected to cyclic loading were cyclically loaded for 30 days. Moreover, Toledano *et al.* [23] reported higher collagen degradation when load cycling was used in demineralized dentin after infiltration with adhesive systems. Such method seems to be not adequate for degradation of demineralized dentin, which was also demonstrated in the present study.

5. Conclusions

The results of the present study provide evidence that endogenous proteases such as MMPs are able to degrade dentin collagen in physiological conditions and that at pH 7.4 the

activity of cathepsins is less relevant than the one of MMPs. This did not occur when the matrices were pretreated with 0.5 M EDC for 60 s. The release of both ICTP and CTX telopeptides fell rapidly over the first four days with or without cyclic stress. Cyclic loading tended to decrease the release of telopeptides. Further investigations are in progress on the role of the pH on both MMPs and cathepsins activity after static and dynamic aging of demineralized dentin.

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