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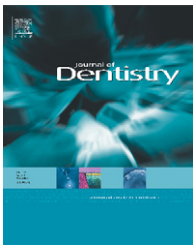

MMP-2 assay within the hybrid layer created by a two-step etch-and-rinse adhesive: Biochemical and immunohistochemical analysis

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ABSTRACT

Objective: Degradation of hybrid layers (HLs) within resin-infiltrated dentine results from multiple degradation factors, including collagenolytic activity of specific matrix metalloproteinases (MMPs). Inhibition of host-derived MMPs may, therefore, slow the degradation of HL. The null hypothesis tested is that the presence of MMP-2 is similar regardless of chlorhexidine (CHX) pre-treatment or the use of an adhesive.

Methods: Powdered dentine prepared from extracted human teeth was divided into 4 groups: (G1) mineralised powder (control group); (G2) dentine powder treated with 1% phosphoric acid for 1 min; (G3) 1% phosphoric acid-etched dentine treated with Adper Scotchbond 1 XT (SB1XT; 3M ESPE); (G4) 1% phosphoric acid-etched dentine treated with 0.2% CHX followed by SB1XT. The concentration of detectable pro-MMP-2 and MMP-2 was assayed using a colorimetric assay system (QuantiSir). In addition, the presence of MMP-2 in the HL was assessed in 1 year-aged adhesive–dentine interfaces using an immunohistochemical approach under FEI-SEM/TEM.

Results: In dentine powder treated with 1% phosphoric acid (G2), MMP-2 level decreased compared to controls (G1); the application of SB1XT (G3) resulted in an increase of MMP-2, whilst 0.2% CHX before SB1XT application (G4), reduced MMP-2. The FEI-SEM/TEM analysis revealed MMP-2 distribution within the HL of aged interfaces showing increase MMP-2 patterns in the control group and minor labelling in the CHX-pretreated specimens.

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Conclusion: The results of this study support the use of non-toxic MMPs inhibitors, such as CHX, as an appropriate additional step in bonding procedures in order to increase the longevity of the adhesive restorations.

1. Introduction

Dentine is a hydrated tissue composed by an intricate three-dimensional organic scaffold embedded with apatite nanocrystals and traversed with microscopic tubules, extending from the pulp chamber to dentine–enamel junction. Approximately 30% of the total volume of the organic matrix of dentine[1] mainly consists of fibrillar type I collagen (90%), in concert with non-collagenous proteins such as glycoproteins, proteoglycans and phosphoproteins.[1] As in other collagen-based tissues, dentine also contains matrix metalloproteinases (MMPs), at least collagenase MMP-8,[2] gelatinases MMP-2 and -9,[3–5] stromelysin MMP-3[6] and enamelysin MMP-20.[2]

MMPs, collectively called matrixins, belong to a multigene family within the metalloproteinase class of endopeptidases. MMPs mediate the degradation of all extracellular matrix molecules, including native and denatured collagen.[7,8] Since increased collagenolytic activity had been observed in carious dentine,[9–11] host-derived MMPs are thought to play a fundamental role in dentine matrix breakdown during caries progression. In addition MMP-2, MMP-8, and MMP-9 have been identified within demineralised dentine lesions *in vitro*.[9]

During restorative procedures, dentine is acid-etched prior to or concomitant to the application of a primer/adhesive system. If maintained in an expanded state, the exposed dentine matrix is impregnated by adhesive monomers forming the so-called hybrid layer (HL). Monomer infiltration is crucial as the instability of the HL is mostly related to inadequate adhesive impregnation of the etched dentine, thus forming sup-optimally infiltrated domains[12,13] that act to initiate failure within the HL. Thus the durability of the adhesive–dentine interface over time relies on the stability of the polymers and the collagen compartment of the HL.

Despite efforts to improve bond stability, recent *in vivo* and *in vitro* studies revealed that HLs are unstable in aqueous environments due to hydrolytic degradation phenomena of both resins[14–16] and collagen fibrils that disappear over time.[14,17–20] It has been speculated that the endogenous MMPs bound to the dentine organic matrix[21] can potentially degrade the exposed collagen fibrils within the HL, if unprotected by adhesive monomers. Since MMPs were shown to be involved in the breakdown of dentine matrix during progression of caries lesions, it is rational to consider that they could also have accounted for the marked disintegration of collagen fibrils recently reported for HL created *in vivo* after 6–14 months of service.[17,19,22]

Indirect evidence of MMP activity was also obtained using chlorhexidine digluconate (CHX), a proven synthetic MMP inhibitor,[23] as a therapeutic primer during the bonding technique. CHX-treated HL preserved the morphological and mechanical properties of HL both under clinical[17,19,22] and *in vitro* conditions.[20] Whilst it is thought that CHX stabilizes resin–dentine bonds due to its inhibitory effect on endogenous dentinal MMPs, direct *in situ* evidence of MMP-2 identification within the HL has not been obtained. Since MMP-2 is present in the highest concentration in the dentine matrix,[5] we chose to examine the distribution of MMP-2 as an example of all endogenous MMPs in the hybrid layer.

The aim of this study was to assay the presence of MMP-2 within HL created by a two-step etch-and-rinse adhesive system *in vitro*, using a correlative immuno-expression/quantification assay and high-resolution SEM/TEM immunohistochemical approach. The tested hypothesis was that the concentration of detectable of host-derived dentinal pro-MMP-2 and MMP-2 is similar regardless of the use of CHX pre-treatment or an adhesive system.

2. Materials and methods

Reagents were purchased from Sigma Chemical (St Louis, MO, USA) unless otherwise specified.

2.1. *QuantiSir analysis*

2.1.1. *Dentine powder preparation*

Twenty extracted sound human third molars were obtained from anonymous subjects following their signed consent under a protocol approved by the University of Trieste. Teeth were stored in 0.5% NaCl containing 0.02% sodium azide at 4 °C for no more than one month. Enamel and roots were cut using a low-speed diamond saw under continuous sterile deionized water irrigation; pulp tissue residuals, if present, were discarded using a hand excavator and predentine layer was removed using a high-speed hand-piece under continuous water irrigation. Dentine powder was then obtained by pulverizing liquid nitrogen-frozen coronal dentine with a steel mortar/pestle (Reimiller, Reggio Emilia, Italy). Aliquots of 250 mg each of dentine powder were obtained and randomly assigned to one of the following treatment groups:

1. *Group 1*: Dentine powder was maintained mineralised as the control group.
2. *Group 2*: Dentine powder was treated with an acid-etchant to simulate the partial demineralisation procedure that is usually performed when using the etch-and-rinse bonding technique. Briefly, the dentine powder was exposed to 1 mL of 1% phosphoric acid for 1 min. Preliminary experiments

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with higher phosphoric acid concentrations (i.e. 10 and 35%) were unsuccessful due to the difficulties in controlling and stopping the demineralising reaction of powderised dentine. The phosphoric acid–dentine powder mixture was stirred continuously with a plastic spatula, adjusted to pH 7.0 with 4 M NaOH and then centrifuged (Eppendorf MiniSpin Plus, Hamburg, Germany) at 14,000 rpm for 2 min. The supernatant was discarded and the acid-etched dentine powder was re-suspended in distilled water and centrifuged for 1 min. The water was discarded, replaced with acetone and the centrifuge process was repeated four times. The etched powder was finally left to air-dry for 12 h at room temperature.

3. *Group 3*: Dentine powder was partially demineralised with 1% phosphoric acid as described for group 2. After rinsing with water, the partially demineralised dentine powder was treated with 400 μL of a two-step etch-and-rinse adhesive system (Adper Scotchbond 1 XT, SB1XT, 3M ESPE, St. Paul, MN, USA). The dentine powder–adhesive mixture was stirred continuously for 90 s with a plastic spatula. Then, the adhesive was extracted from the acid-etched dentine with 500 μl of acetone under centrifugation at 14,000 rpm for 1 min. The acetone extraction procedure was repeated four times. After removing the acetone, the treated dentine powder was allowed to air-dry for 12 h at room temperature.
4. *Group 4*: Dentine powder was partially demineralised with 1% phosphoric acid as described above, rinsed with water, centrifuged and then the powder was treated with a water solution of 0.2% CHX for 1 min. The partially demineralised dentine powder was centrifuged, the supernatant was removed and the precipitate was treated with SB1XT and subsequent extraction protocols, as described for Group 3.

2.1.2. *Assay for MMP-2*

To quantify the presence of MMP-2 in the untreated (Group 1) and treated dentine powder (Groups 2–4), the QuantiSir™ gene knockdown assay system (Epigentek, New York, USA) was used according to the manufacturer's instructions. In the assay, the substrate lysates containing the targeted protein (MMP-2) are stably spotted on the specifically treated micro-wells with unique protein capture buffer. The spotted protein can be then recognized with the target-specific antibody and spectrophotometrically measured through detection anti-body–chromogen reaction system. The human recombinant MMP-2 proenzymes (Cat. No. PF023) and anti-MMP-2 antibody (Cat. No. IM33) used in the study were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany) whilst the Horseradish peroxidase-conjugated anti-mouse secondary antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA).

In detail, after treating the 4 groups of dentine powder as previously described, protein extracts were obtained using the extraction buffer (Q1) and were then diluted with the protein capture buffer (Q3) at a 1:1 ratio and 10 μL of the diluted protein extract was added into central area of each strip well in the 96-well plate of the QuantiSir kit. The strip wells were then incubated at 37 °C (with no humidity) for 90 min. 150 μL of Q4 blocking buffer were added to the dried wells and subsequently incubated at 37 °C for 30 min. After 3 rinses with Q2 wash buffer, 50 μL, of the primary antibody (diluted to 1 μg/mL in Q5 buffer) were added to the centre of each strip and wells were incubated at room temperature for 60 min on an orbital shaker. Strip wells were then washed again 4 times and incubated with the secondary antibody (1:1000) for 30 min at room temperature.

After 5 washes in Q2 buffer, 100 μL of Q6 developing solution were added to the wells and incubated at room temperature for 5 min in the dark. Colour development was monitored and the reaction was stopped by adding 50 μL of Q7 to the wells.

Negative controls were performed using (1) buffer solution only; (2) buffer solution and primary antibody only; (3) buffer and secondary antibody only.

The absorbance value of each well was read on a Minireader at 450 nm (Biorad, Segrate, Milano, Italy) and the absorbance values, as well as the amount of MMP-2 detected (ng/μL) were calculated from the standard curve of MMP-2. All experiments were run in triplicate ($N = 3$) and repeated three times. As the values were normally distributed (Kolmogorov–Smirnof test), data were analysed with a one-way ANOVA and Tukey's post hoc tests.

2.2. *Immunohistochemical analysis of hybrid layer*

2.2.1. *Specimen preparation*

Twelve intact sound human third molars were selected after patient obtaining informed consent under a protocol approved by the Human Assurance Committee of the University of Bologna, Italy. Teeth were stored in 0.5% NaCl containing 0.02% sodium azide at 4 °C for no more than one month. Mid-coronal dentine was exposed with a low speed diamond saw under water irrigation (Micromet, Remet, Bologna, Italy) and 1 mm-thick middle/deep dentine disks were created. A standardized smear layer was created on the exposed coronal dentine with wet 180-grit wet silicon carbide paper.

The exposed dentine surfaces of all dentine disks were acid-etched with 35% phosphoric acid for 15 s (Etching Gel, 3M ESPE), rinsed with water, gently air-dried and kept moist in order to remove excess water without drying the dentine in accordance with the wet-bonding technique, until adhesive was applied according to the manufacturer's instructions. Specimens were equally and randomly assigned to two treatment groups ($N = 6$):

Table 1 – Composition of Adper Scotchbond 1XT bonding system tested in the study.

Adhesive	Composition
Adper Scotchbond 1XT (3M ESPE)	Etchant
	35% phosphoric acid gel
	Bonding
	2-Hydroxyethylmethacrylate (HEMA)
	Polyalkenoic acid copolymer
	Bis-phenol A diglycidylmethacrylate (Bis-GMA)
	Water–camphorquinone
	Ethanol

1. *Group 1*: Acid-etched specimens were treated with a water solution of 0.2% CHX for 30 s (continuous scrubbing on the surface with a micro-brush for 30 s), gently air-dried in accordance with the wet bonding technique, then bonded with SB1XT (CHX group) and light-cured; composition of SB1XT adhesive system is reported in Table 1.
2. *Group 2*: Specimens were acid-etched, rinsed and bonded with SB1XT (control group) and light-cured.

A 1-mm thick layer of flowable composite (Filtek Flow, 3M ESPE) was placed on the resin-hybridized dentine surfaces and light-cured for 20 s (Curing Light 2500, 3 M EPSE). The dentine–adhesive–composite interface was exposed by trimming the specimens and obtaining 1 mm-thick beams in accordance with the non-trimming microtensile testing technique. Exposed interfaces were stored for 12 months (T_{12}) in artificial saliva containing 0.02% NaN_3 at 37 °C. After storage, the HL of CHX-treated and control specimens were processed for immunohistochemical analysis and submitted to either a pre-embedding or a post-embedding immunohistochemical technique.

2.2.2. *Pre-embedding technique and FEI-SEM tissue processing*

Pre-embedding immunolabelling technique of the exposed bonded interfaces was performed in accordance with Breschi et al.[24] using a mouse IgG anti-human MMP-2 (Abcam, Cambridge, UK – Cat. No. Ab1818) as monoclonal primary antibody.

Briefly, resin-bonded specimens were immersed in 0.05 M Tris–HCl buffer solution (TBS) at pH 7.6, pre-incubated in normal goat serum (British BioCell International, Cardiff, UK) in 0.05 M TBS at pH 7.6 for 30 min, then incubated overnight with the primary antibody anti-MMP-2 at 4 °C. Gold labelling was performed using a goat anti-mouse IgG conjugated with 15 nm colloidal gold particles (British BioCell International) as secondary antibody in 0.02 M TBS at pH 8.2 for 90 min at room temperature. Specimens were then rinsed with 0.02 M TBS at pH 8.2, washed in water, fixed in 2.5% glutaraldehyde for 4 h and rinsed with 0.15 M cacodylate buffer pH 7.2. After dehydration in ascending ethanol concentrations, specimens were dried using hexamethyldisilasane and coated with carbon using a Balzers Med 010 Multicoating System (Bal-Tec AG, Liechtenstein). Specimen analysis was performed using FEI-SEM (JSM 890, JEOL Ltd., Tokyo, Japan) at 7 kV and 1×10^{-12} A. Images were obtained with a combination of back-scattered and secondary electron modes.

2.2.3. *Tissue processing for TEM and post-embedding technique*

Bonded resin/dentine interfaces assigned to the post-embedding technique were dehydrated in graded concentrations of ethanol and embedded in LR White resin (London Resin, Berkshire, UK). Semi-thin sections (1 μm) were cut with glass knives on a Reichert Jung Ultracut E ultramicrotome. Selected areas of the 1 μm thick sections were trimmed for ultra-thin sectioning and 80 nm thick sections were prepared and mounted on formvar carbon-coated nickel grids.

Grid-mounted tissue thin sections were processed for the immunohistochemical labelling following a post-embedding technique in accordance with Breschi et al.[25] Similarly to the previously described pre-embedding procedure, immunolabelling was performed after overnight incubation of specimens with the primary anti-MMP-2 antibody in 0.05 M TBS at pH 7.6, followed by the colloidal gold-conjugated secondary antibody in 0.02 M TBS at pH 8.2.

Grids of undemineralised and unstained specimens were examined under TEM (Philips CM-10) operating at 70 kV.

3. Results

3.1. *QuantiSir analysis*

Means and standard deviation of MMP-2 absorbance and levels recovered in all groups are listed in Table 2. Statistical analysis revealed significant differences amongst all groups ($p < 0.05$). In dentine powder treated with 1% phosphoric acid (Group 2), MMP-2 levels decreased 43% compared to levels obtained in mineralised dentine (control group – Group 1). After the application of SB1XT on acid-etched dentine (Group 3), MMP-2 detected by the specific antibody increased significantly (280%), whilst 0.2% CHX pre-treatment before SB1XT application on acid-etched dentine (Group 4), reduced the MMP-2 levels by 27% compared to the SB1XT treatment alone (Group 3).

Negative controls showed no reaction.

3.2. *Immunohistochemical analysis of the hybrid layer*

As seen by FEISEM and TEM, positive immunolabelling patterns for MMP-2 were identified due to the presence of the gold nanoparticles conjugated with the secondary antibodies. The presence of MMP-2 appeared as spherical spots of approximately 15 nm in diameter either electron-reflective (white spots, FEI-SEM) or electron-dense (black spots, TEM).

Table 2 – Means and standard deviation of recorded MMP-2 level of absorbance and corresponding MMP-2 concentration of the four tested groups.

Group	MMP-2 (A-450 nm)	MMP-2 (ng/μL)
Group 1: mineralised dentine	0.892 ± 0.048^{a}	8.3 ± 0.4^{A}
Group 2: 1% H_3PO_4 demineralised dentine	0.505 ± 0.014^{b}	4.7 ± 0.1^{B}
Group 3: 1% H_3PO_4 demineralised dentine + SB1XT	1.412 ± 0.016^{c}	13.1 ± 0.1^{C}
Group 4: 1% H_3PO_4 demineralised dentine + 0.2% CHX + SB1XT	1.028 ± 0.032^{d}	9.5 ± 0.3^{D}

Values are mean ± standard deviation. Groups with different superscripts are statistically significant ($p < 0.05$).

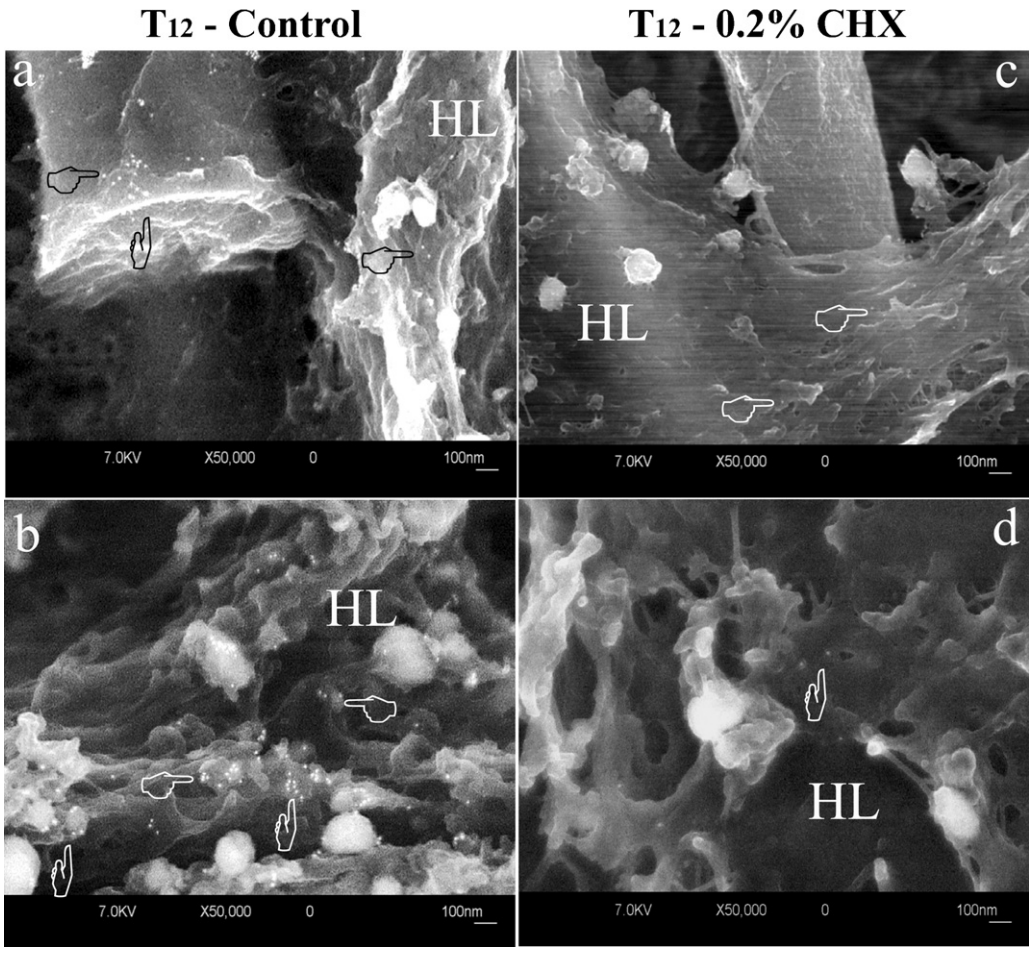

Fig. 1 – FEI-SEM micrographs showing the adhesive interfaces created by Scotchbond 1XT (after 1 year of storage in artificial saliva) then incubated with anti MMP-2 primary antibody followed by a secondary antibody conjugated with gold nanoparticles (15 nm in diameter). Gold particles appear as spherical electron reflective (white) structures (pointers). Almost no labelling was visible on mineralised dentine since MMP-2 epitopes were masked by the hydroxyapatite. (a and b) Hybrid layer (HL) created by Adper Scotchbond 1XT applied in accordance with manufactures' instructions (control; i.e. no chlorhexidine pre-treatment was used) showing clusters of nanoparticles (pointers) linked to MMP-2. (c and d) Hybrid layer (HL) created after etching and 0.2% chlorhexidine application showing scarce labelling linked to MMP-2 (pointers).

Differences of MMP-2 labelling were detected between control mineralised specimens (Figs. 1a, b and 2a, b) and adhesive interfaces that were pre-treated with CHX as a therapeutic primer (Figs. 1c, d and 2c, d) after 12 months of *in vitro* ageing in artificial saliva (T_{12}). Both FEI-SEM (pre-embedding protocol) and TEM (post-embedding protocol) analysis revealed MMP-2 labelling throughout the HL of control specimens, whilst CHX-treated specimens revealed a scarce and scattered labelling.

Higher magnification FEI-SEM images revealed the presence of gold nanoparticles identifying MMP-2 in the intertubular matrix and along the surface of resin tags (Fig. 1b and d). Additionally the formation of small aggregates of MMP-2 was visible both on the dentine matrix and in correspondence of the resin tags or resin fragments under FEI-SEM (Fig. 1b). Similar features were also detectable within HL under TEM (Fig. 2b). Conversely, CHX pre-treated and aged specimens revealed scarce MMP-2 labelling and absence of enzymes aggregates regardless of the pre- or post-embedding immunohistochemical protocol (Figs. 1d and 2d).

4. Discussion

The biochemical and immunohistochemical evidence of the present study demonstrated for the first time that MMP-2 is entrapped within the HL created by a two-step etch-and-rinse adhesive system and that the amount of MMP-2 available for detection varies as a function of dentine treatment. Although the presence of MMP-2 has been identified irrespective of the use of an adhesive system, its expression varied significantly depending on the step of the bonding procedure (i.e. etching, priming-and-bonding) and on pre-treatment or not with CHX. These results therefore support the rejection of the tested hypothesis.

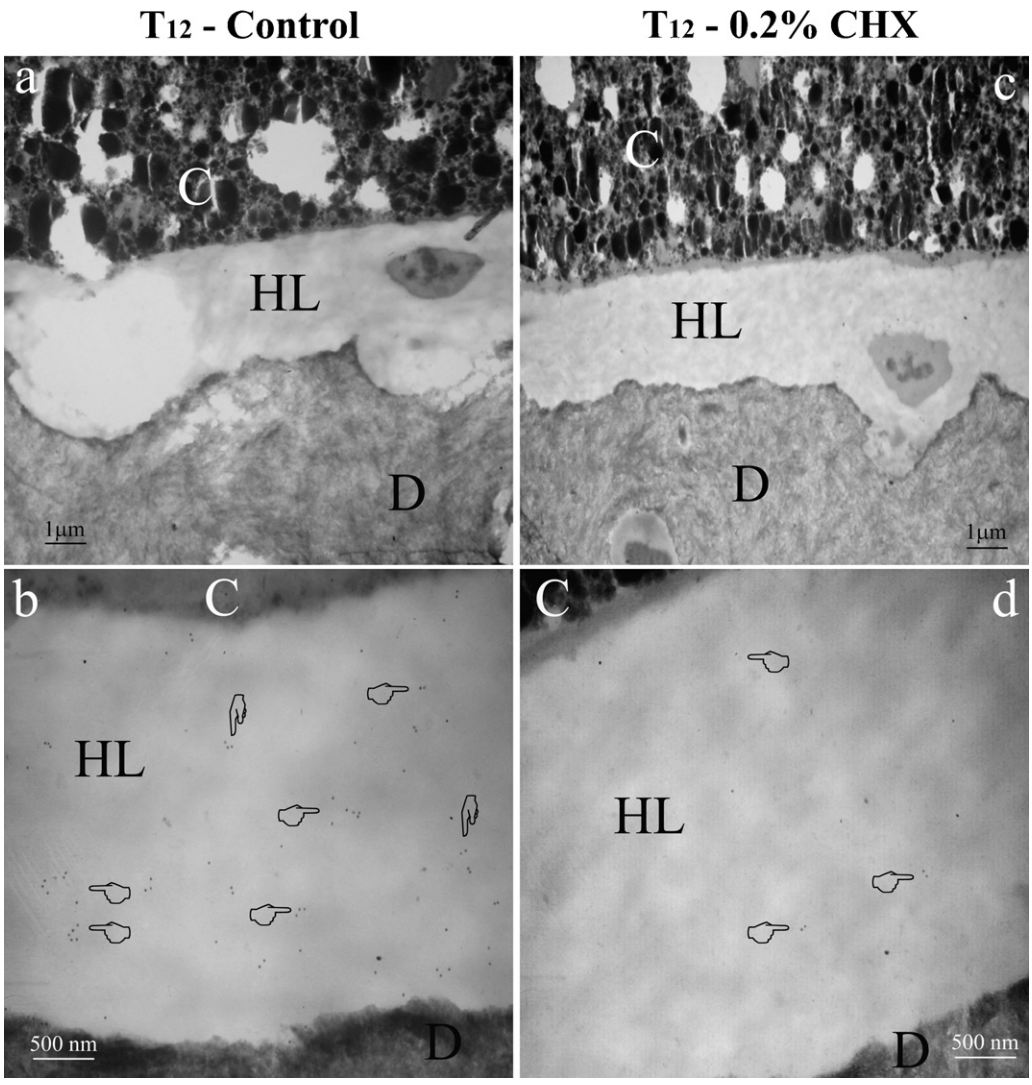

Fig. 2 – TEM micrographs showing the adhesive interfaces created by Adper Scotchbond 1XT (after 1 year of storage in artificial saliva) then incubated with anti-MMP-2 primary antibody followed by a secondary antibody conjugated with gold nanoparticles (15 nm in diameter). Gold particles appear as spherical electron dense (black) structures (pointers). Almost no labelling was visible on mineralised dentine since MMP-2 epitopes were masked by the hydroxyapatite. Low (a) and higher (b) magnification views of adhesive interface created by Adper Scotchbond 1XT applied in accordance with manufactures' instructions (control, i.e. no chlorhexidine pre-treatment was used). At higher magnification view (b) MMP-2 labelling (pointers) is clearly evident, mainly as small clusters of nanoparticles within the hybrid layer (HL). There are 78 gold nanoparticles in the control HL. Dentine (D); composite (C). Low (c) and higher (d) magnification views of adhesive interface created by Adper Scotchbond 1XT applied on etched and 0.2% chlorhexidine treated dentine. At higher magnification view (d), scarce labelling (pointers) identifying MMP-2 was detected within the hybrid layer (HL). Dentine (D); composite (C). There are only 13 gold nanoparticles in the CHX-treated HL.

Previous studies investigated the presence and distribution of MMP-2, -3, -8 and -9 in human coronal[2,3,5,6] and radicular[26] dentine. As MMP-2 has been shown to be extremely robust, resisting acidic and thermal denaturation,[2] it was speculated that MMP-2 might maintain its activity even after the completion of clinical bonding procedures. Whilst the different components of adhesive systems (i.e. etchants, solvents and resin monomers) may potentially inhibit the activity of dentinal MMPs,[27] the proteolytic activity of partially demineralised dentine that was previously observed,[4,27] might be due to proteolytic enzymes that remain embedded in the underlying mineralised dentine matrix and that would be slowly released over time.[27]

The fluorescent enzyme assays that were previously used to screen the relative proteolytic activity in adhesive-treated acid-etched dentine[4,28] revealed an intrinsic proteolytic activity. However, this proteolytic activity could not be related with a specific protease. Preliminary zymography and western-blot findings indicate that activated isoforms of MMP-2 are present together with latent forms of MMP-2 in extracts prepared from freshly extracted human dentine.[5] The correlative biochemical and immunohistochemical approach

used in the present study, clarified the active involvement of MMP-2 in hybrid layer degradation.

The reduction of MMP-2 observed for the acid-etched compared to the mineralised dentine specimens may be related to acid mediated extraction, or structural changes/denaturation of the enzyme. Indeed the antigenicity of a single protein as revealed by its specific binding to a monoclonal antibody provides definitive evidence of an optimal conservation of the epitope structure.[29,30] As phosphoric acid (1%) has a pH value of 1.0, the acidic conditions could unfold and denature a number of protein/enzymes within the dentine matrix and reduce the antibody binding.[31,32] Although an exact description of the denatured states cannot always be specified,[33] any condition where the physical and chemical properties of the protein are different from the designated native state is called a non-active state. Protein/enzyme denaturation may cause profound changes, especially in secondary and tertiary structure of a protein at a level it cannot be recognized with a conventional antibody using immunohistochemical techniques.[34] Indeed the monoclonal antibodies used in the present study are highly sensitive,[30] and the protocol employed needs optimal preservation of the epitope for antigen–antibody binding.

It is notable that the treatment of the phosphoric acid-etched dentine with an etch-and-rinse adhesive (i.e. SB1XT) increased MMP levels about 3.5 times compared to the phosphoric acid-etched dentine (Table 2). A potential explanation for this finding would be that the treatment with the slight acidic adhesive system might have demineralised additional underlying mineralised dentine and activated the uncovered. As the pH of SB1XT is around 3.3–3.5,[4] we speculate that its acidity is sufficient to demineralise additional dentine. On the other hand, as the same amount of protein extracts was loaded on each well, we hypothesize that the two-step etch-and-rinse system was able to increase the relative MMP-2 extraction within each dentine protein aliquots.

We speculate that the same chain of events that were considered for the phosphoric acid-etched adhesive-treated dentine also occurred for dentine that was acid-etched and treated with CHX and adhesive. The phosphoric acid would denature some of the MMP-2, whilst the treatment of acid-etched dentine with the etch-and-rinse adhesive (SB1XT) would attack underlying mineralised dentine exposing new MMP without causing significant denaturation. The treatment with CHX may have inhibited only the MMP-2 exposed by 1% phosphoric acid, but not that of MMPs subsequently exposed by SB1XT etching since the CHX was already bound to the demineralised matrix (Table 2). Due to its substantivity and inhibitory capacity on MMPs, CHX could have altered the protein conformation by hiding some enzyme's epitopes, thereby reducing the probability of MMP-2 to be recognized in the immunohistochemical assay.[35,36] As previous studies have clearly demonstrated the improved preservation of the HL integrity and strength,[19,20] this masking of the epitope is most likely also related to the long-term or inhibition of MMP-2 functional activity in the adhesive–dentine interface.

The immunolabelling of HL along with the FEI-SEM/TEM immunolocalization and immuno-quantification of MMP-2 in mineralised dentine, phosphoric acid-etched dentine and adhesive-treated acid-etched dentine supports the hypothesis that MMP-2 may be one of the dentine-bound MMPs involved in HL degradation.

In conclusion, the current study supports the possible role of endogenous dentine MMP-2 on *in vitro* and *in vivo* degradation of collagen fibrils within HL, when acid-etched dentine matrices are infiltrated with simplified etch-and-rinse adhesives.[17,19,20,22] Ultimately, the final consequence of this degradation may be the loss of continuity between collagen fibrils in the HL and the underlying mineralised matrix into which they are attached, which has the potential to eventually lead to lower bond strengths, increased nanoleakage and dentine/pulpal sensitivity. For these reasons the use of non-toxic MMPs inhibitors, such as CHX, may be considered as an appropriate additional step in bonding procedures. MMP inhibitors may contribute to HL stability over time, which, in turn, may increase the longevity of the adhesive restorations. Long-term clinical trials will need to be conducted in order to verify this notion.

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