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# Chlorhexidine stabilizes the adhesive interface: A 2-year *in vitro* study

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ABSTRACT

*Objectives.* This study evaluated the role of endogenous dentin MMPs in auto-degradation of collagen fibrils within adhesive-bonded interfaces. The null hypotheses tested were that adhesive blends or chlorhexidine digluconate (CHX) application does not modify dentin MMPs activity and that CHX used as therapeutic primer does not improve the stability of adhesive interfaces over time.

*Methods.* Zymograms of protein extracts from human dentin powder incubated with Adper Scotchbond 1XT (SB1XT) on untreated or 0.2–2% CHX-treated dentin were obtained to assay dentin MMPs activity. Microtensile bond strength and interfacial nanoleakage expression of SB1XT bonded interfaces (with or without CHX pre-treatment for 30 s on the etched surface) were analyzed immediately and after 2 years of storage in artificial saliva at 37 °C.

*Results.* Zymograms showed that application of SB1XT to human dentin powder increases MMP-2 activity, while CHX pre-treatment inhibited all dentin gelatinolytic activity, irrespective from the tested concentration. CHX significantly lowered the loss of bond strength and nanoleakage seen in acid-etched resin-bonded dentin artificially aged for 2 years.

*Significance.* The study demonstrates the active role of SB1XT in dentin MMP-2 activation and the efficacy of CHX inhibition of MMPs even if used at low concentration (0.2%).



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## 1. Introduction

Despite successful immediate bonding, the longevity of the adhesive interface remains questionable due to physical (occlusal forces, expansion and contraction stresses related to temperature changes) and chemical factors challenging the adhesive interface [1,2]. The hybrid layer created by two-step etch-and-rinse adhesives containing high percentages of hydrophilic monomers, results in the formation of a porous bonded interface [3] that behaves as a permeable membrane [4] allowing elution of unreacted monomers, water sorption, polymer swelling and resin hydrolysis [5,6]. In addition, enzymatic activity can degrade the exposed type I collagen fibrils located at the bottom of the hybrid layer due to the activation of endogenous collagenolytic factors previously identified within the dentin organic matrix [7–9].

These enzymes belong to the family of matrix metalloproteinases (MMPs) and are involved in connective tissue turn-over, degrading almost all extracellular matrix components [10,11]. Recent findings indicate that MMP-2, -3, -8, -9 and -20 are present within the human dentin matrix [12–16].

Although these enzymes are involved in dentinogenesis and in caries progression mechanisms [12,17], the role of dentin MMPs in mature dentin is still unclear. Human dentin matrices exhibit variable collagenolytic and gelatinolytic activities when mixed with dentin/enamel bonding agents with different pHs [8,9]. Thus, the simple application of adhesive systems on acid-etched dentin substrate can activate dentinal MMPs, initiating autolytic phenomena that will eventually affect the hybrid layer.

Chlorhexidine digluconate (CHX) has the ability to inactivate MMP-2, -8 and -9 [18]. Despite the use of CHX as a therapeutic primer to stabilize the adhesive interface over time [19–22], the relationship between the collagenolytic activity of dentin, MMPs role in hybrid layer degradation, and the mechanism of CHX inhibition of MMPs needs to be clarified. Aqueous 2% CHX prevented much of the decline in bond strength and increased nanoleakage seen both *in vitro* and *in vivo* in deciduous and permanent teeth [19–21]. Additionally, a recent *in vitro* study confirmed the protective role of 0.2% CHX after 1 year of storage of bonded specimens in artificial saliva, suggesting that lower concentration of CHX can be equally effective compared to 2% concentrations [22].

The purpose of this study was to evaluate the effect of 0.2 and 2% CHX on the adhesive–dentin interfaces created by a two-step etch-and-rinse adhesive system. The null hypotheses tested were that (1) dentin MMPs activity is not affected by adhesive or CHX application and (2) the use of CHX as a therapeutic primer does not affect bond strength and interfacial nanoleakage expression after 2 years.

## 2. Material and methods

### 2.1. *Zymographic analysis*

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

Ten freshly extracted human molars were selected after patient's informed consent was obtained under a protocol approved by the University of Trieste. Enamel, roots and remnant pulp tissue were removed and dentin powder was obtained by pulverizing liquid nitrogen-frozen coronal dentin with a steel mortar/pestle (Reimiller, Reggio Emilia, Italy). Five aliquots of 1 g each of dentin powder were obtained and treated as follow. Group 1: untreated mineralized dentin powder; Group 2: dentin powder demineralized in 1% aqueous $H_3PO_4$ for 10 min; Group 3: mineralized dentin powder treated with 3 mL of Adper Scotchbond 1XT (SB1XT, 3M ESPE, St Paul, MN, USA) for 24 h at 4 °C in dark condition; Groups 4 and 5: mineralized dentin powder was treated either with 0.2 or 2% CHX water solution (Groups 4 and 5, respectively) for 30 min at 4 °C, rinsed with 1 mL of distilled water (five times), then incubated with 3 mL of SB1XT for 24 h at 4 °C in dark condition. All specimens were thoroughly rinsed with 4 mL of acetone and then centrifuged for 10 min (14,000 rpm) at 4 °C. Specimens were then re-suspended in 4 mL extraction buffer (50 mM Tris–HCl pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100), 0.1% non-ionic detergent P-40 (0.1 mM $ZnCl_2$, 0.02% $NaN_3$) and EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) for 24 h. Specimens were then centrifuged, supernatants were collected and protein content was precipitated with 25 wt% trichloroacetic acid (TCA) at 4 °C. TCA precipitates were re-solubilized in loading buffer (25% Trizma–HCl 1 M pH 6.8, 8% (w/v) sodium dodecyl sulfate, 40% glycerol and distilled water).

Total protein concentration of the extracts of mineralized and partially demineralized dentin powder was determined using the Bradford assay. Proteins were electrophorized under non-reducing conditions on 7.5% SDS-polyacrylamide gels copolymerized with 2 g/L gelatin (porcine skin). Activation of gelatinase proforms was achieved with 2 mM p-aminophenylmercuric acetate (APMA) for 1 h at 37 °C and then incubated for 24 h at 37 °C in zymography buffer ($CaCl_2$, NaCl and Tris–HCl, pH 8.0). Gels were stained in 0.2% Coomassie Brilliant Blue R-250 and destained in destaining buffer (50% methanol, 10% acetic acid, 40% water).

Control zymograms were incubated in the presence of 5 mM EDTA and 2 mM 1,10-phenanthroline to inhibit gelatinases.

### 2.2. *Specimens preparation for microtensile bond strength test*

An additional 48 non-carious extracted human molars were selected and flat surfaces of middle/deep dentin were exposed with a slow speed diamond saw (Micromet, Bologna, Italy). Smear layer-covered dentin surfaces were etched with 35% phosphoric acid for 15 s (etching gel, 3M ESPE), rinsed and surfaces were blot dried according to the wet bonding technique. Specimens were randomly assigned to the following treatments ($N = 16$ teeth in each group). Groups 1 and 2: acid-etched dentin surfaces were treated with aqueous solutions of either 0.2 or 2% CHX, respectively, for 30 s, blot dried and bonded with SB1XT; Group 3: received no pre-treatment before SB1XT application (control). SB1XT was applied in accordance with manufacturers' instructions and light-cured. Resin com-

posite build-ups were created with Filtek Z250 (3M ESPE, St Paul, MN, USA).

### 2.3. Microtensile bond strength evaluation

Resin–dentin sticks with cross-sectional area of approximately 0.9 $mm^2$ were obtained in accordance with the non-trimming technique [23]. Each stick was measured and recorded for bond strength calculation. Sticks were divided in two equal groups and either stored for 24 h ($T_0$) or for 2 years ($T_{2\,years}$) in artificial saliva (prepared in accordance with the protocol of Pashley et al. [7], but without protease inhibitors) at 37 °C. Sticks were stressed until failure with a simplified universal testing machine at a crosshead speed of 1 mm/min (Bisco Inc., Schaumburg, IL, USA). Failure modes were evaluated as described by Breschi et al. [22].

### 2.4. Statistical analysis

As values were normally distributed (Kolmogorov–Smirnof test), data were analyzed with a two-way ANOVA (tested variables were: CHX concentration, time of storage) and Tukey's post hoc test. To analyze the effect of chlorhexidine on fracture modes, mixed and dentin cohesive failures were combined. Wilcoxon Signed Ranks Test was used to analyze the differences in failure modes between $T_0$ and $T_{2\,years}$ for each group, and Kruskal–Wallis test was used to compare the fracture modes between the groups within each time points. Statistical significance was set at $p < 0.05$.

### 2.5. Nanoleakage evaluation

Twelve additional teeth (N = 4/group) were prepared and bonded as previously described. Specimens were then vertically cut into 1-mm thick slabs to expose the bonded surfaces and submitted to the two storage times in artificial saliva at 37 °C: $T_0$ and $T_{2\,years}$. Specimens were then submerged in 50 wt% ammoniacal silver nitrate for 24 h, rinsed, photodeveloped and processed for nanoleakage analysis under TEM and examined under TEM (Philips CM-10) operating at 70 kV [24].

## 3. Results

### 3.1. Zymographic analysis

No enzymatic activity was found in proteins extracted from mineralized dentin (Group 1, Fig. 1, Lane 1). Zymograms of $H_3PO_4$-demineralized dentin extracts (Group 2) showed multiple forms of gelatinolytic enzymes (Fig. 1, Lane 2), with the 66-kDa as MMP-2 active form, a fainter band of 86 kDa corresponding to the active form of MMP-9 and other minor gelatinolytic bands with lower molecular weights (Fig. 1, Lane 2). Zymograms of SB1XT-treated dentin (Group 3) showed an intense band at 66-kDa identified as MMP-2 active form, a 72-kDa band corresponding to the proform of MMP-2 and a fainter band at 86-kDa corresponding to a slight activity of MMP-9 active form. In addition, lower molecular weight bands were detected (Fig. 1, Lane 3). Incubation of mineralized dentin powder with 0.2 or 2% CHX solutions followed by SB1XT application resulted in complete inhibition of both MMP-2 and -9 activity (Fig. 1, Lanes 4 and 5).

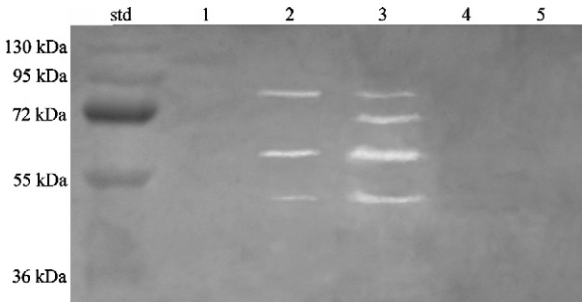


**Fig. 1 – Gelatin zymogram of MMPs from dentin extracts after sonication, TCA precipitation, resuspension and activation with 2 mM APMA for 1 h. Molecular masses, expressed in kDa, are reported in Std lane. *Lane* 1: Absence of any gelatinolytic activities in proteins extracted from mineralized dentin powder; *Lane* 2: Identification of MMP-2 and -9 isoforms in $H_3PO_4$-treated dentin; *Lane* 3: MMPs isoforms detected in mineralized dentin powder after incubation with SB1XT for 24 h produced an increase in gelatinolytic activity of MMP-2 and a decrease of MMP-9 compared to the $H_3PO_4$-demineralized dentin; *Lanes* 4 and 5: Incubation with 0.2 or 2% CHX respectively and SB1XT, produced complete inhibition of all forms of MMP-2 and -9 activity.**

Control zymograms incubated with 5 mM EDTA and 2 mM 1,10-phenanthroline showed no enzymatic activity (data not shown).

### 3.2. Microtensile bond strength and fracture mode analyses

Mean bond strength obtained at $T_0$ and $T_{2\,years}$ and failure mode distributions are summarized in Table 1. At $T_0$, there were no differences between the bond strength of control versus the two CHX-tested concentrations (0.2% CHX = 39.2 ± 9.3 MPa; 2% CHX = 41.2 ± 9.6 MPa; control = 40.8 ± 8.7 MPa; $p > 0.05$). Conversely, after 2 years of *in vitro* storage ($T_{2\,years}$) the bond strengths of SB1XT-control group decreased approximately 67% (control = 13.4 ± 4.9 MPa), while in CHX-treated groups the decrease of bond strength was limited between 16 and 30%, depending on whether the acid-etched dentin was primed with 0.2 or 2% CHX, respectively (0.2% CHX = 32.6 ± 8.3 MPa; 2% CHX = 28.5 ± 7.2 MPa). Fracture mode analysis did not demonstrate any statistically significant differences between the groups or within the groups at any time points (Table 1).

### 3.3. Interfacial nanoleakage analysis

In CHX-treated adhesive interfaces, minor scattered silver particles were observed, with no large-scale silver grain accumulation (Fig. 2). In control specimens immersed in silver nitrate there was marked uptake of silver into the middle of the hybrid layer that was manifested as a large cluster of black silver grains (Fig. 3).

**Table 1 – Bond strengths of control (no CHX-treated) versus 0.2 and 2% CHX-treated specimens tested immediately ($T_0$) or after 2 years ($T_{2\,years}$) of aging.**

| | Microtensile bond strength of Adper Scotchbond 1XT | Failure mode (%) | | | |
|---|---|---|---|---|---|
| | | A | CD | CC | M |
| Control $T_0$ | $40.8 \pm 8.7^{a}$ (8) [6/184] | $40^{A}$ | 0 | 0 | $60^{A}$ |
| Control $T_{2\,years}$ | $13.4 \pm 4.9^{c}$ (8) [5/180] | $35^{A}$ | $20^{B}$ | 0 | $45^{A}$ |
| 0.2% CHX $T_0$ | $39.2 \pm 9.3^{a}$ (8) [6/178] | $40^{A}$ | $15^{B}$ | 0 | $45^{A}$ |
| 0.2% CHX $T_{2\,years}$ | $32.6 \pm 8.3^{a,b}$ (8) [4/169] | $40^{A}$ | 0 | 0 | $60^{A}$ |
| 2% CHX $T_0$ | $41.2 \pm 9.6^{a}$ (8) [6/175] | $35^{A}$ | 10 | 0 | $55^{A}$ |
| 2% CHX $T_{2\,years}$ | $28.5 \pm 7.2^{b}$ (8) [3/164] | $30^{A}$ | $25^{B}$ | 0 | $45^{A}$ |

Values are mean ± SD (number of teeth) in MPa (number of premature failed sticks/number of intact sticks tested). Groups identified by different lower case letters are significantly different ($p < 0.05$). Groups identified by the same uppercase letters are not significantly different ($p > 0.05$). Failed sticks were not included in statistical analyses. Distribution of failure mode (in %) among tested groups in the different periods of analysis is also reported. A: adhesive; CD: cohesive failure in dentin; CC: cohesive failure in resin composite; M: mixed failure, as described by Breschi et al. [22].

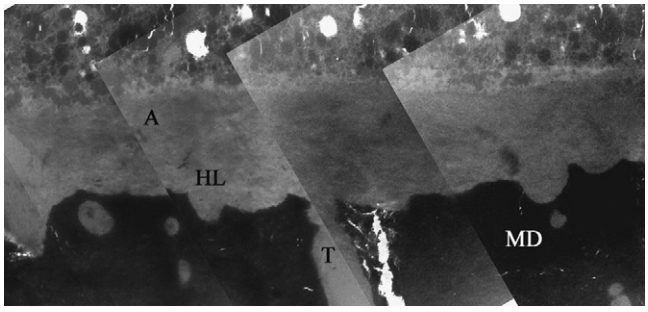


**Fig. 2 – TEM image obtained combining numerous micrographs of a representative specimen treated with 0.2% CHX for 30 s, then bonded with SB1XT and stored for 2 years in artificial saliva at 37 °C. The adhesive (A) interface revealed only very few scattered particles of silver nanoleakage within the hybrid layers (HL). MD = mineralized dentin; T = dentinal tubules; A = filled adhesive. Bar = 2 μm.**

## 4. Discussion

The results of this study showed that the application of SB1XT to mineralized dentin powder for 24 h activates dentin MMP-2, while pre-treatment of the demineralized dentin powder with 0.2 or 2% CHX inhibits that enzyme activity as assayed by the zymographic analysis. In addition, 0.2 or 2% CHX used as a therapeutic primer on acid-etched dentin preserved the resin–dentin bond strength over time by significantly reducing the rate of decrease if bond strength over 2 years. CHX-treated specimens showed higher bond strength and a higher quality of the hybrid layer revealed as a lower interfacial nanoleakage expression compared to control specimens. These results support the rejection of both tested null hypotheses.

Previous studies demonstrating the CHX-related improvement in long-term stability of hybrid layer integrity and bond strength only hypothesized that the effect would be based on MMP-2, -8, -9 inhibition by CHX [18–21]. The assumption is logical, since those MMPs are the main matrix-degrading enzymes in dentin [14,15,25]. Even though the quantitative analysis of different MMPs in dentin remains to be completed, the currently available data indicate that MMP-2 may be the prevalent MMPs in dentin [14,15,25].

The present data provides for the first time direct evidence of endogenous dentin-bound MMPs (especially MMP-2) activation due to adhesive application and adhesive-activated enzyme inhibition due to CHX, as assayed by zymography. This evidence confirms that the increase in dentin matrix-degrading activity by adhesives [8,9] can be related to MMP-2

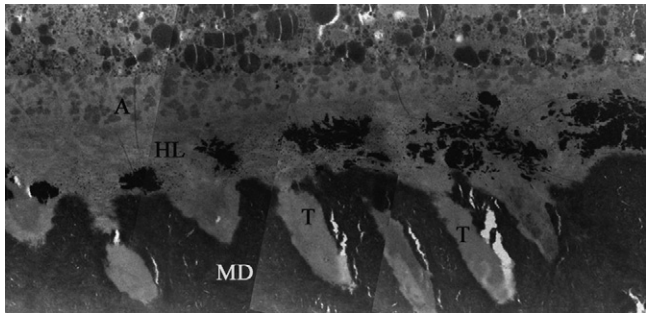


**Fig. 3 – TEM image obtained combining numerous micrographs of a representative control specimen bonded with SB1XT and stored for 2 years in artificial saliva at 37 °C. This control adhesive interface reveals extensive interfacial silver nanoleakage due to individual silver grains and large clusters of silver deposits within the collagen fibrils of the hybrid layer (HL). Abbreviations are same as in Fig. 2. Bar = 2 μm.**

activation, as well as the involvement of MMP-2 in hybrid layer degradation and loss of bond strength [19–21].

The reduction of MMP-9 in zymography observed with SB1XT treatment compared to the $H_3PO_4$-demineralized dentin is somewhat surprising, especially in relation to the apparent increase in MMP-2 activity. However, previous studies demonstrated differences in gelatinase inhibition that may be related to the gelatinase fibronectin-like domain [26,27]. Gelatinases (MMP-2 and -9) demonstrate unique structural similarity that makes them different from other MMPs, namely fibronectin-like type II modules in catalytic domains. These modules form collagen-binding domains (CBDs), which interact specifically with gelatin, being critical for the positioning of substrates for cleavage [26,27]. In spite of the close functional properties of MMP-2 and -9 CBDs [28], they may have different sensitivities to inhibitors. For example, dimethyl sulfoxide inhibits MMP-2 by affecting CBD-2 mediated interaction of MMP-2 with gelatin [27,29] that is not seen with MMP-9 in spite of 90% reduction in gelatin binding [26]. This difference has been suggested to be related to the differences in gelatinase CBDs between MMP-2 and -9 [28]. In another study, slight modifications of a synthetic MMP inhibitor molecule (acyclic N-arylsulfonyl homocysteine hydroxamate) caused significant changes in inhibitory activity, the relative $IC_{50}$ values varying from 0.5- to 30-fold between MMP-2 and -9, with inhibition of MMP-9 being most effective with different variations among five MMPs tested (MMP-1, -2, -3, -9 and -13) [30]. Alternatively, the MMP-9-specific inhibition may be related to the MMP-9 hemopexin unit, which differs from MMP-2 with the presence of a long hinge region. Competitive inhibition experiments using recombinant MMP-9 hemopexin domains demonstrate significant (60–71%) inhibition of MMP-9 in zymographic analysis, presumably due to decreased binding to gelatin [31].

The loss of hybrid layer integrity and increased nanoleakage after aging for 2 years correlated well with previous studies [2]. In contrast, the absence of nanoleakage in CHX-treated specimens also correlates well with previous studies demonstrating well-preserved hybrid layer treated either with CHX or storage in mineral oil replacing water to prevent enzymatic hydrolysis of collagen [32,33]. Together with the marked activation of MMP-2 by the SBIXT adhesive and inhibition of gelatinolytic activity by CHX (both observed in zymographic analysis, Fig. 1), the preservation of hybrid layer integrity and bond strength provide definitive proof that MMPs are behind the time-dependent destruction of hybrid layer, and that MMP inhibition offers a valuable tool for improving the durability of composite dentin bonding.

In conclusion, this study clarifies the active role of dentin MMP-2 in hybrid layer degradation and supports the use of CHX as additional primer due to its inhibiting effect on dentin MMP-2 activity.

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