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Chlorhexidine preserves the hybrid layer *in vitro* after 10-years aging

ABSTRACT

Objectives. The present study investigated the ability of a chlorhexidine (CHX)-containing primer (0.2% aqueous solution) to inhibit dentinal enzymes, preserve the hybrid layer (HL) and remain within the HL, after 10 years of aging in artificial saliva at 37°C.

Methods. Non-carious extracted molars were assigned to two groups, cut into slabs exposing middle/deep dentin, etched and bonded with Adper Scotchbond 1XT (SB1XT) with or without 0.2% CHX aqueous solution pretreatment. Composite build-ups were made, and the specimens were cut in 1-mm thick bonded sticks. *In-situ* zymography was performed on freshly prepared specimens (T0) and specimens aged for 10 years (T10-yr) at 37°C in artificial saliva, to investigate endogenous gelatinolytic activity within the HL. At T10yr, specimens were also decalcified and embedded in epoxy resin for TEM analysis. Micro-Raman spectroscopy was performed at T0 and T10-yr to evaluate the chemical profiles in intertubular dentin and the HL.

Results. *In-situ* zymography showed less pronounced enzymatic activity in the CHX-pretreated group ($p < 0.05$) regardless of aging, maintaining a similar level of fluorescence at T0 and T10-yr ($p > 0.05$). TEM results showed that 98% of the HL had been degraded in the control group, while 95% of the HL was intact in the experimental group. Moreover, all the Raman spectra peaks assigned to CHX could be identified only in the CHX-pretreated group (T0 and T10-yr).

Conclusion. *In vitro*, CHX remains in the HL after 10 years with its inhibitory effect preserved. This may be the underlying factor for HL preservation after this long aging period.

Chlorhexidine preserves the hybrid layer *in vitro* after 10-years aging

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INTRODUCTION

The ability of endogenous proteases such as matrix metalloproteinases (MMPs) present in dentin to accelerate the degradation of the dentinal organic matrix, including the incompletely-infiltrated collagen fibrils at the bottom of the hybrid layer (HL), has been well documented and extensively investigated. Several approaches have been developed to enhance the durability of HL [1–4].

Among those, various agents that inhibit or inactivate MMPs have been introduced [5–13]. To date, the largest number of studies is available using chlorhexidine (CHX) as the MMP inhibitor. This is probably because of the availability of CHX and its widespread use in the dental practice. There is ample *in vitro* and *in vivo* research data that shows at most part a beneficial effect of CHX on HL longevity and bond strength [14–25]. Although the results are promising, evidence that MMP inhibition can last over a long time, such as five or ten years, are scarce and conflicting. A research group has followed patients with class V restorations with or without a CHX-containing primer for a period of 3 years and found no differences in the survival of the restoration between the groups [26]. In contrast, Loguercio et al. [25] demonstrated in an *in vitro* study that higher bond strength is associated with CHX-treated specimens after 5-year aging, compared with the control. This is so far the longest aging time described in the literature for CHX inhibition of dentin MMPs. In that study, CHX ions were found to remain within the hybrid layer after 5 years.

Accordingly, the objective of the present study was to investigate whether CHX retains its MMP-inhibitory properties, preserves the HL, and whether it was still present in the HL after 10 years of aging in artificial saliva. The null hypotheses tested were: 1) CHX does not retain its MMPs inhibitory properties after 10-year aging in artificial saliva; 2) CHX has not effect on preservation of the HL after 10-year aging in artificial saliva; 3) CHX is no longer present in the HL after 10 years of aging in artificial saliva.

MATERIALS AND METHODS

Sample preparation

One-millimeter-thick dentin slabs derived from middle/deep dentin were obtained from extracted human sound third molars using a low-speed saw (Micromet, Remet, Bologna, Italy) with water-cooling (N=8 teeth per group). The teeth were either used immediately after extraction or kept frozen (-20°C) for up to one month prior to use. The teeth for the baseline (T₀) study and the 10-year aging (T_{10-yr}) part of the study were prepared at two different time points in an identical way as follows: A standardized smear layer was created on each slab using 240-grit wet silicon carbide paper. The slabs were randomly assigned to 2 main groups. Group 1 was etched for 15 s with a 35% phosphoric acid-etching gel (3M ESPE; St Paul, MN, USA). After thorough rinsing, the dentin was treated with a 0.2% CHX gluconate aqueous primer for 30 s. The 0.2% CHX gluconate aqueous solution used in this study was prepared from the 20% stock solution (Mondial Snc, Limena, Padova, Italy) by the pharmacy of the University hospital in Trieste, Italy. After the pretreatment, the dentin slabs were gently blot-dried and left moist for wet-bonding, according to a previously established protocol [18,20]. The Adper Scotchbond 1XT (SB1XT, 3M ESPE) adhesive was applied according to the manufacturer's instructions and polymerized using a quartz-tungsten-halogen light-curing unit (Curing Light 2500; 3M ESPE) for 20 s. A 1-mm composite build-up was created using flowable composite (Filtek 250 Flow; 3M ESPE) and light-cured for 40 s. In group 2 (control), the dentin slabs were etched, wet-bonded with SB1XT and built up with composite, as in group 1, without using the CHX primer. All the bonding procedures were performed by two expert operators. The bonded dentin slabs were cut into 1-mm thick specimens under water irrigation. The specimens for the 10-yr study were stored for 10 years in the artificial saliva (pH 7.4) produced in accordance with Pashley et al. (without the protease inhibitors) [27] at 37°C (T_{10-yr}). During the entire process of sample aging, the artificial saliva was changed with a fresh

solution in 2-week intervals. The specimens for the baseline study were prepared from freshly extracted teeth (or teeth kept frozen for up to one month prior to use) right prior to the completion of the 10-year aging of the other half of the specimens, so that the experiments could be performed on all the specimens at the same time using the same equipment. The bonded specimens from each tooth were randomly distributed between three experiments: *in situ* zymography, transmission electron microscopy (TEM) and Micro-Raman spectroscopy. The TEM analysis was performed only for the aged specimens.

In-situ zymography

In-situ zymography was performed following a previously-reported protocol (Mazzoni et al. 2012; 2014). Briefly, each specimen was glued to a microscope slide to expose the resin-dentin interface and ground down to ~50 μm thick. The specimen was covered with diluted self-quenched fluorescein-conjugated gelatin (E-12055; Molecular Probes, Eugene, OR), protected with a glass cover-slip and kept overnight in a dark humid chamber at 37 °C. After incubation, the microscopic slides were examined using confocal laser scanning microscopy (excitation/emission wavelength: 488/530 nm; TCS SP2 AOBS, Leica Microsystems GmbH, Wetzlar, Germany). Several images were obtained from each specimen. Each image was created as a stack of images from the superficial layer of the specimen downward at 1 μm thick intervals. Relative differences between the groups in the enzymatic activity, represented by the fluorescence within the HL, were quantified as the integrated density of the fluorescence signals using ImageJ software (National Institutes of Health, Bethesda, MD). A rectangular selection was created, saved and used for all the measurements in the experiments in order to standardize the measurements. The intensity of the signal was measured on three different sites on each image.

Statistical analysis

Because the *in-situ* zymography data was normally distributed, analysis was performed using two-way ANOVA and Tukey post-hoc tests to examine the effects of “CHX pre-treatment” and “aging” on potential gelatinolytic activities. For all tests, statistical significance was pre-set at $\alpha = 0.05$. Analyses were performed using Stata 12.0 (StataCorp, College Station, Texas).

Transmission electron microscopy

Specimens that had been aged for 10 years were completely demineralized in 0.1 M formic acid/sodium formate (pH 2.5). This was followed by fixation in Karnovsky’s fixative (2.5 wt% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L cacodylate buffer; pH, 7.3) for 8 h. The samples were post-fixed in 1% osmium tetroxide for 1 h. The decalcified specimens were dehydrated in ascending concentrations of ethanol (50%-100%), immersed in propylene oxide as a transition medium and embedded in pure epoxy resin. Thin sections (90 nm) were cut and mounted on formvar-coated single-slot copper grids. The grids were stained with 2% uranyl acetate and Reynold’s lead citrate and examined with TEM (JEM-1230, JEOL, Tokyo, Japan) at 110 kV.

Micro-Raman spectroscopy

Identification of CHX along the resin-dentin interface was performed using a micro-Raman spectrometer/microscope (Horiba Scientific Xplora, Villeneuve d’Ascq, France) with a 638-nm diode laser through an X100/0.9 NA air objective. Raman signals were acquired using a 600-lines/mm grating centered between 400 and 2200 cm^{-1} . The parameters employed were 100 mW, 30 s accumulation time with 5 co-additions. Spectra were taken at three random sites in intertubular dentin, starting from the top of the HL to the underlying dentin at 1- μm thick intervals across the HL/dentin interface. Eleven spectra were acquired for each site using a computer-controlled x-y-z stage.

RESULTS

Results of *in-situ* zymography were evaluated qualitatively (Fig. 1) and quantitatively (Fig. 2). The highest enzymatic activity appeared to be concentrated in the HL and the dentinal tubules underneath the HL in all specimens. Distribution of areas containing high fluorescence signals differed among the groups examined. At T₀, these areas extended ~10 µm into the hybrid layer and the underlying dentin. At T_{10-yr}, 15-20 µm thick, high-intensity fluorescence signals were identified in the CHX-treated group and the control group (Fig. 1). In some of the aged specimens, discreet bubble-shaped defects were seen in the adhesive layer and resin composite, probably attributed to hydrolytic degradation.

At T₀, pronounced enzymatic activity was identified in the HL of the control group, after density quantification of the fluorescence signals. In contrast, enzymatic activity was significantly lower (28%) in the CHX-pretreated groups ($p < 0.05$). After aging for 10 years, there was a 40% lower level of fluorescence in the experimental group compared with the control group ($p < 0.05$). There was no significant difference in the fluorescence intensity of the CHX-treated groups at T₀ and at T_{10yr} ($p > 0.05$).

After aging for 10 years, TEM examination showed that 95% of the HL in the CHX+SB1XT group were intact (Fig. 3d,e,f). In the 5% of the hybrid layer that had degraded (marked with an asterisk in Fig. 3d), the collagen fibrils were sparse and had partially degraded microfibrillar strands and short chains shown by the open arrowheads in Fig. 3f. The hybrid layer was ~3 µm thick. In the SB1XT group without CHX treatment, 98% of the hybrid layer had been degraded after aging for 10 years (Fig. 3a-c). Only the resin tags linked the adhesive and composite with the underlying intertubular dentin. Thin layers of collagen marked with black arrows remained at the top (Fig. 3b) and bottom (Fig. 3c) of the partially-degraded hybrid layers.

Representative Raman spectra of the experimental groups are shown in Fig. 4. Raman

spectra within the 400-2200 cm^{-1} region contained bands associated with adhesive resin, dentin minerals, collagen and CHX. The most prominent active peaks that characterize the adhesive resin were located at 1113 cm^{-1} (C-O-C), 1454 cm^{-1} (CH_2), 1608 cm^{-1} , 1638 cm^{-1} (C=C) and 1720 cm^{-1} (C=O). The characteristic peaks attributed to mineralized dentin could be identified at 960 (PO_4^{-3}) and 1070 cm^{-1} (CO_3^{-2}). Those attributed to the collagen were found at 1242/1280 cm^{-1} (amide III), 1452 cm^{-1} (CH_2) and 1667 cm^{-1} (amide I). These peaks were observed in all the experimental groups. However, the peaks that are assigned to CHX, at 670 cm^{-1} (C-Cl), 1268 and 1564/1608 cm^{-1} (unprotonated/protonated bands associated to CHX, respectively) [30] could be identified in the CHX+SB1XT group only.

Apart from the presence of the aforementioned bands, the Raman chemical profile revealed interesting differences between the groups. For all groups, the top spectra refer to the HL, which could be identified because of the presence of the adhesive and collagen peaks, and the abrupt reduction of mineral peaks. The results are indicative of the removal of minerals from this region during acid-etching. In the SB1XT group without CHX treatment, spectral changes with noises and broadening of the amide III and I peaks were identified after 10 years. In the CHX+SB1XT group, characteristic spectra of the HL were well-defined from the 1st to the 8th μm . More intense vibrations in the 670 cm^{-1} region (black pointer) were identified when compared to the control group (SB1XT). These vibrations could probably be attributed to the C-Cl bands of CHX. Another characteristic peak attributed to CHX (1564 cm^{-1} ; green pointer) was also identified in the CHX+SB1XT group [25,30]. Other atypical vibrations were identified in the 1268 cm^{-1} region (red pointer) throughout the entire spectra, which were exclusively seen in the CHX+SB1XT group.

Raman signals representative of the dentin substrate could be detected after the 8th μm , as indicated by increases in the intensity of the mineral peaks (PO_4^{-3} and CO_3^{-2}). Nevertheless, C=C vibrations (1608 cm^{-1}) remained in these spectra. The CHX contains two basic -CNH-

NH-CNH- groups, both of which readily accept a proton to form a protonated species with several tautomeric forms [30]. During the interaction of CHX with demineralized dentin, protonation of the amide groups of CHX with the carboxylic acid/hydroxyl functional groups of dentin occurs. The tautomeric forms resulted from protonation could be identified by the additional peak at 1268 cm^{-1} , as well as the previously-demonstrated peak at 1608 cm^{-1} [30]. However, the 1608 cm^{-1} peak was overlapped with other peaks that are commonly identified in methacrylate adhesive systems.

DISCUSSION

The present study demonstrated that CHX ions deriving from the additional primer used during bonding procedures was still present within the HL after 10 years of aging in artificial saliva. In addition, MMP-inhibitory properties within the HL were retained and integrity of the HL was preserved 10 years after priming the acid-etched dentin with CHX. These results warrant rejection of all three null hypotheses.

Chlorhexidine has come a long way since the late 40s, when it was first investigated for its antimicrobial properties. The biguanide finds application in caries prevention and periodontal disease therapy in the 70s [31]. A subsequent milestone was reached when degradation of collagen was reported to be caused by the immunological responses of an organism, particularly involving the MMPs [32,33]. The use of different agents to inhibit these collagen-degrading enzymes gradually picked up interest [34]. It was only natural to investigate whether CHX also exhibits these properties because it was widely used in clinical practice. Gendron et al. [35] were the first to report that CHX inhibits several MMPs even in very low concentrations. Following this, Pashley et al. [27] translated this interesting concept to restorative dentistry, as MMPs play an important role in caries progression [36]. The authors found that collagen degeneration could be prevented after application of CHX to acid-etched dentin. After these pivotal early works, CHX has been extensively tested as the inhibitor of

dentinal MMPs. As mentioned above, the beneficial effect of CHX has been reported in numerous studies. However, given that the majority of the aforementioned studies had a short follow-up/aging time, it is not known whether this protective effect of CHX will last, and if so, for how long. Sadek et al. [37] hypothesized that CHX leaches out of the HL after 18 months, while other authors have shown that CHX ions were retained in HLs after 2 [38] or 5 years [25] of *in vitro* aging of resin-dentin bonds.

Results of the present study demonstrated that at least some CHX ions were retained within the HL after 10 years of artificial aging. The retained CHX ions either maintained their initial inhibitory effect or exerted continuous inhibitory effect and contributed to the preservation of the integrity of the HL. In the control group of the aged specimens, gelatinolytic activity was significantly higher, possibly promoting collagen degradation within the water-rich, resin-sparse regions of the HLs. This was confirmed with TEM and regions of degradation corresponded to Raman spectral changes (broadening of amide III and I peaks) that were indicative of damage of the collagen fibrils. This is because the amide peaks III and I are sensitive to the molecular conformation of polypeptide chains [39–41]. The CHX-pretreated group had a stable level of gelatinolytic activity at the baseline and after 10 years. Interestingly, there was also no statistically significant difference in the gelatinolytic activity between the baseline and aged control group, although the activity was 21% higher in the aged group, possibly due to a relatively high standard deviation. One of the possible explanations could be that the MMPs are not entirely free, but are attached to the collagen fibrils by their fibronectin and hemopexin binding sites, so they can cleave a limited amount of the surrounding collagen molecules, which leads to lower enzymatic activity after the surrounding substrate runs out [42,43]. However, with the leaching out of the resin monomers, new fibrils are denuded and a fresh set of MMPs is available for collagen degradation. This is why the authors indeed believe that the initial onset of the collagen degradation driven by the MMPs is a very important event

that starts a continuous degradation process, and if the onset can be prevented, the long term prognosis for the survival of composite restorations could be significantly improved. These results add substantially to the knowledge on the long-term effects of CHX and further reinforce the importance of MMP inhibition in the preservation of the HL. The results represent the longest *in vitro* aging time available in the literature so far.

Although the anti-MMP effect of CHX has been demonstrated for 20 years, the mechanisms responsible for this property have not been fully elucidated. Several mechanisms have been proposed to account for the affinity of CHX toward the dentin substrate. Chlorhexidine is a strong base and has a cationic structure at physiological pH. Hence, CHX induces cationic-anionic reaction with the organic and mineral components of dentin. The reaction with organic components is presumed to involve the carboxylic and hydroxyl groups of collagen and noncollagenous phosphoproteins [44]. For carbonated apatite, the inorganic component in dentin, CHX binds presumably to the mineral phosphates [45]. In addition, CHX removes zinc and calcium ions necessary for the activity of MMPs and also reacts with the essential sulfhydryl groups and/or cysteine in the catalytic site of these enzymes [35,46]. Chlorhexidine also demonstrates substantivity to oral tissues. Carrilho et al. compared the substantivity in three dental substrates, mineralized, partially-demineralized and completely-demineralized dentin. The authors found that CHX has the best substantivity to partially-demineralized dentine at a concentration of 0.2% [47]. In such a substrate, CHX probably exerts its affinity toward the extracellular organic matrix, as well as the carbonated apatite in the underlying mineralized dentin. In the present study, the dentin was partially-demineralized, possibly contributing to the prolonged effects of CHX. Interestingly, micro-Raman spectroscopy indicated that the CHX peaks may represent the tautomeric form that results from protonation of the amide groups of CHX with the carboxylic acid/hydroxyl groups in dentin

[30]. This further reinforces the hypothesis that interaction of CHX with dentin is more than merely electrostatic in nature.

Interpretation of the results of the present study should be limited to 2-step etch-and-rinse adhesive systems. The interaction of adhesive resins with dentin differs significantly between the etch-and-rinse and the self-etch adhesives. In the self-etch mode, demineralization and resin infiltration occur simultaneously. In contrast, a separate etching step is employed in the etch-and-rinse systems. Etching is the initial point that demineralizes the dentin, leaving denuded collagen fibrils surrounded by water on the bottom of the hybrid layer. It is the hydrolysis by host derived proteases in the presence of water that degrades the collagen fibrils and facilitates the degradation of the adhesive resin and leaching out of the unpolymerized monomers from the resin [3,4]. As the monomers leach out of the hybrid layer, they unveil other collagen fibrils and their enzymes join the reaction, entering a vicious cycle and rendering the degradation process slow and continuous. Another factor probably contributing to the degradation onset and propagation is the presence of a certain percentage of hydrophilic monomers (HEMA) within the Scotchbond 1XT adhesive blend used in this study. These monomers are added in a vast majority of contemporary dental adhesives in order to improve the hybridization of the hydrophobic adhesive resin and hydrophilic dentin collagen fibrils. However, this can result in incomplete polymerization and consequent leaching out of the monomers, and water sorption by the adhesive resin [48]. In fact, the *in situ* zymography in the present study demonstrated a low amount of fluorescence emission within the resin composite, only in the aged groups. As the tested specimens were of 1 mm thickness, there was a large surface of each specimen exposed to the influence of artificial saliva at 37°C for 10 years, which indeed lead to accelerated aging, degradation of both dentin organic matrix and adhesive resin, and water sorption by the composite resin. We speculate that due to these reasons the

uptake of the gelatin digested by the dentinal enzymes occurred, showing fluorescence on the composite resin surface.

Because the other available long-term aging study also investigated 2-step etch-and-rinse adhesives [25], further long-term aging research on CHX should be directed toward other adhesive systems. Nevertheless, the use of a separate CHX-containing aqueous primer may be recommended to clinicians in daily practice. Such a procedure involves a simple additional step in the adhesive procedure, but produces excellent long-term results in HL integrity and bond strength preservation in the etch-and-rinse systems.

CONCLUSIONS

Chlorhexidine demonstrates anti-proteolytic effect and protection of the hybrid layer from degradation after 10 years of accelerated aging. The results further reinforce the importance of MMP inhibition for the preservation of the integrity of the resin-dentin interface. Future studies should validate the present results using CHX-containing adhesives.

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FIGURE LEGENDS

Figure 1. Resin-dentin interfaces incubated with quenched fluorescein-labeled gelatin. (a) Image acquired in the green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created with SB1XT without CHX pre-treatment at T₀; (b) Image of SB1XT without CHX pre-treatment, obtained by merging the differential interference contrast (DIC) image (showing optical density of the resin-dentin interface) and the image acquired in the green channel; (c) Image acquired in green channel of the HL created by the application of SB1XT to acid-etched dentin with CHX pre-treatment at T₀, showing lower level of fluorescence; (d) Image of HL created with SB1XT without CHX pre-treatment obtained by merging the DIC image and image acquired in the green channel at T₀; (e) Image acquired in green channel, showing fluorescence in the HL created with SB1XT without CHX pre-treatment at T_{10-yr}; (f) Image of SB1XT with CHX pre-treatment at T_{10-yr}, obtained by merging the DIC image and image acquired in green channel; (g) Image acquired in green channel of the HL created by the application of SB1XT to acid-etched dentin with CHX pre-treatment at T_{10-yr} showing lower level of fluorescence compared to the control group; (h) Image of HL created with SB1XT with CHX pre-treatment obtained by merging the DIC image and image acquired in the green channel at T_{10-yr}; SB1XT: Adper Scotchbond 1 XT; CHX: chlorhexidine D: dentin; HL: hybrid layer; R: resin composite.

Figure 2. Quantification of the gelatinolytic activity within the resin-dentin interfaces of the tested groups. Groups marked with asterisks are significantly different ($p < 0.05$).

Figure 3. Transmission electron microscopy images of the resin-dentin interface. Specimens were completely-demineralized and sections were stained intact after 10 years of water storage. Upper row: specimens bonded without a CHX-containing separate primer (SB1XT). Lower row: specimens bonded with the application of a CHX-containing separate primer (CHX +SB1XT). Bar widths a) 1 μm ; b) 500 nm; c) 500 nm; d) 1 μm ; e) 500 nm f) 100 nm

Abbreviations - H: hybrid layer; A: adhesive; C: resin composite; D: intertubular dentin; T: dentinal tubule; **Symbols** - Asterisk: collagen fibrils within the hybrid layer that degraded completely (a) or partially (d); Black arrows: a thin layer of collagen that remained at the top (b) and bottom (c) of the partially-degraded hybrid layers; Open arrowheads: (e) polyalkenoic acid copolymer component of the adhesive, (f) the open arrowheads represent a high magnification of the asterisked region in Figure d where in the collagen fibrils have unraveled and degraded into microfibrils.

Figure 4. Micro-Raman line-spectra acquired at the adhesive-dentin interface created by SB1XT without CHX (SB1XT) and with a CHX primer (CHX+SB1XT). The relative peaks associated with methacrylate monomers, mineralized and demineralized dentin in the hybrid layer were observed in both groups. The peak of chlorhexidine diacetate was only evident in the CHX+SB1XT groups (T_0 and $T_{10\text{yr}}$).

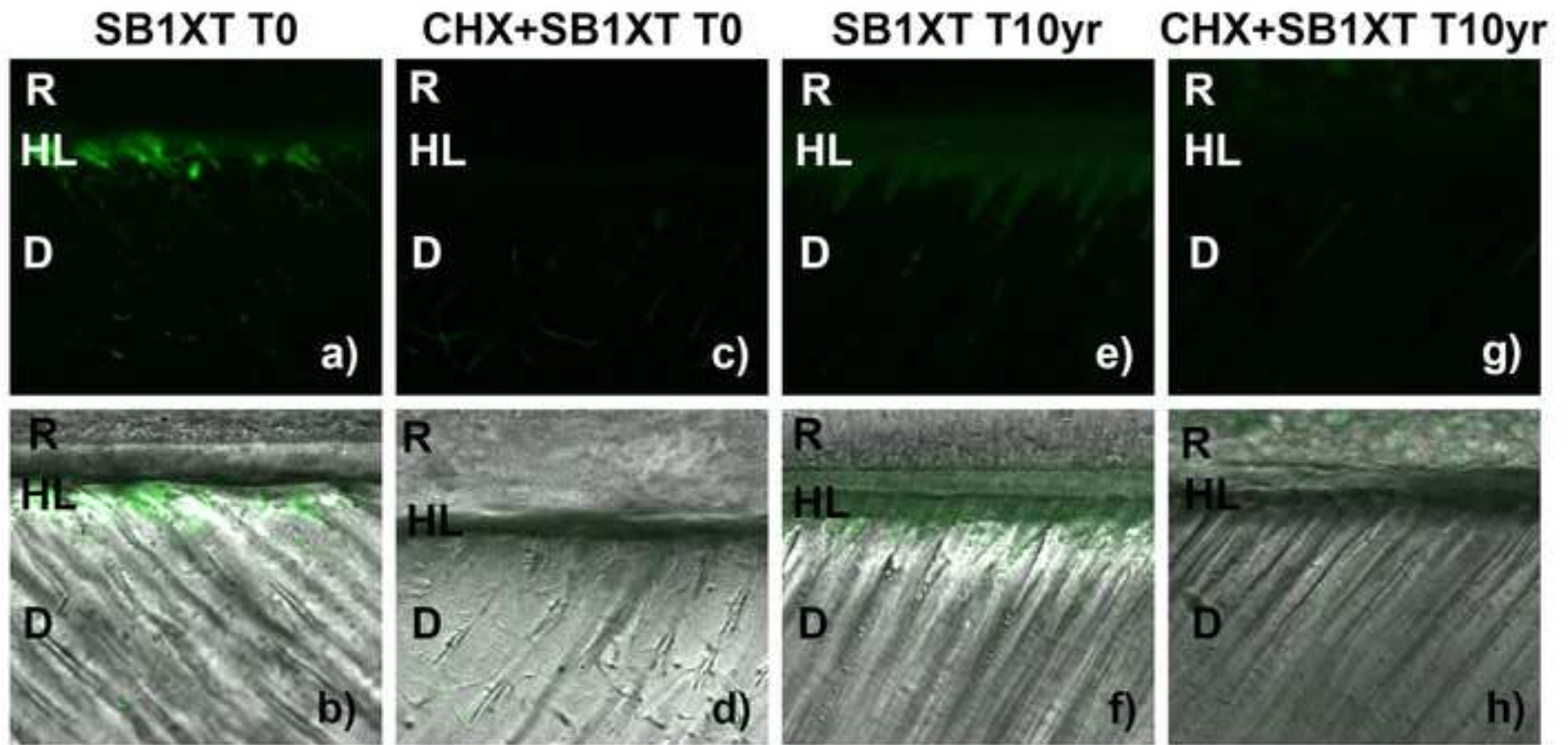
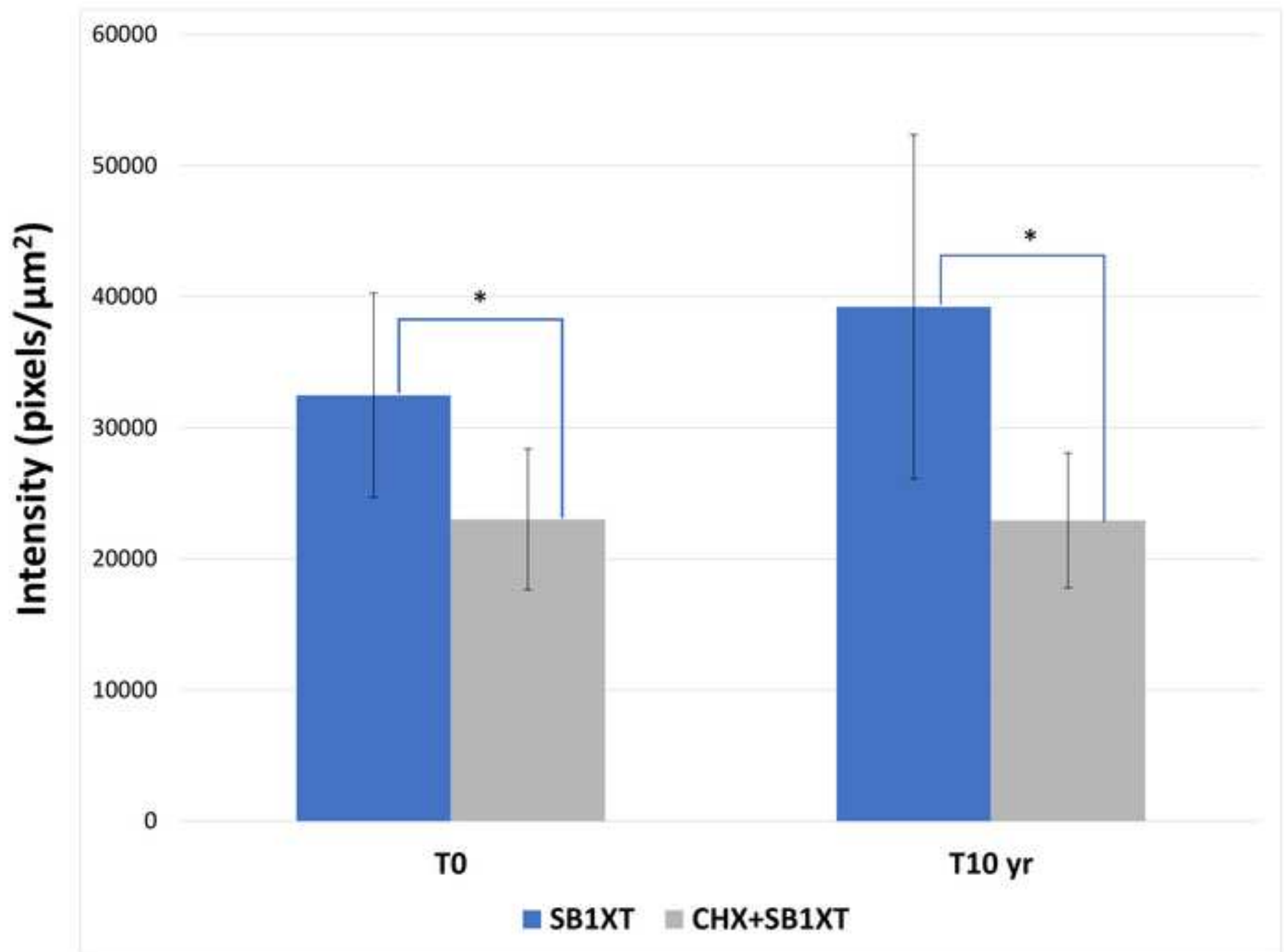
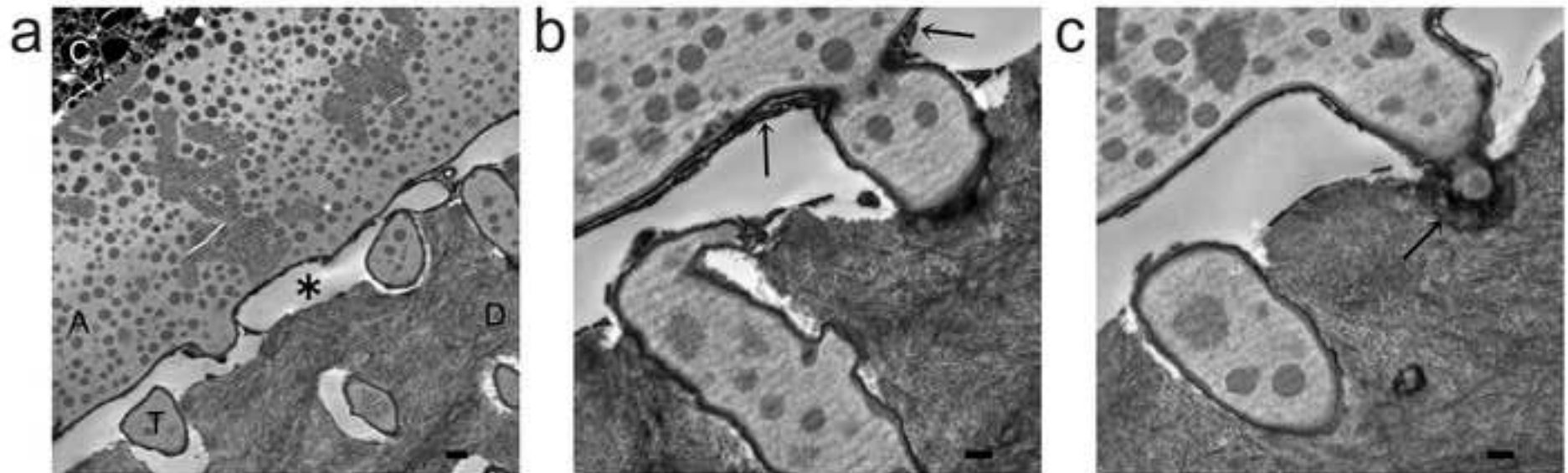


Figure 2. Quantification of the gelatinolytic activity within the resin-dentin interfaces of the tested groups. Groups marked with asterisks are significantly different ($p < 0.05$).

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SB1XT



CHX+SB1XT

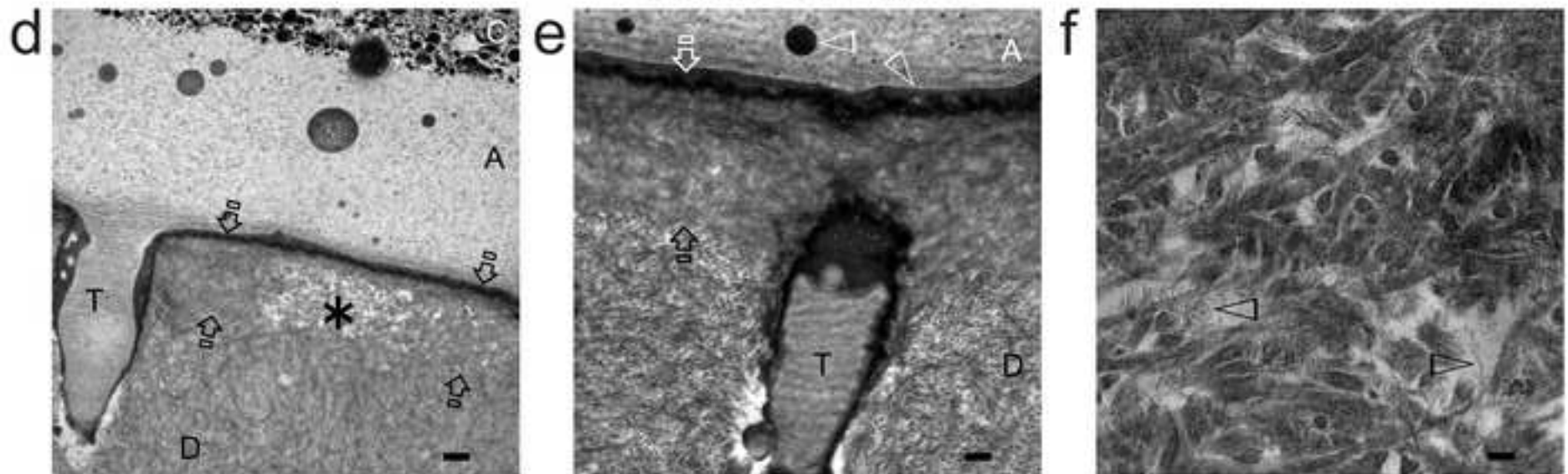


Figure 4. Micro-Raman line-spectra acquired at the adhesive-dentin interface created by SB1XT without CHX (SB1XT) and with a CHX primer (CHX+SB1XT).

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