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Effect of Minocycline on the Durability of Dentin Bonding Produced with Etch-and-Rinse Adhesives

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Clinical Relevance

Chemical-modified tetracyclines can be considered an alternative to retard the degradation of dentin-bonding interfaces.

SUMMARY

Objectives: To evaluate the effect of minocycline and chlorhexidine pretreatment of acid-etched dentin on the longevity of resin-dentin bond strength (μTBS) and nanoleakage of two-step etch-and-rinse adhesives.

Methods: Before application of Prime & Bond NT and Adper Single Bond 2 in occlusal dentin, the dentin surfaces were treated with 37% phosphoric acid, rinsed, air-dried, and rewetted with water (control group), 2% minocycline, or 2% chlorexidine digluconate. Composite buildups were constructed incrementally, and specimens were longitudinally sectioned to obtain bonded sticks (0.8 mm^2) to be tested in tension (0.5 mm/min) immediately or after 24 months of water storage. For nanoleakage, two specimens of each tooth/period were immersed in the silver nitrate solution, photo-developed, and polished with SiC paper for analysis under energy-dispersive X-ray spectroscopy/scanning electron microscopy.

Results: Reductions of the μTBS and increases in the nanoleakage were observed for both

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adhesives when the rewetting procedure was performed with water. Stable bonds were observed for the 2% minocycline and 2% chlorexidine digluconate groups after 24 months.

Conclusions: The use of 2% minocycline as pretreatment of acid-etched dentin is one alternative to retard the degradation of resin-dentin interfaces over a 24-month period as well as 2% chlorexidine digluconate.

INTRODUCTION

In spite of the high immediate resin-dentin bonds created by simplified etch-and-rinse adhesives, dentin adhesive interfaces suffer substantial deterioration after water aging.[1,2] It is hypothesized that this biodegradation involves a cascade of events,[3] starting with the incomplete infiltration of the exposed collagen fibril matrix after acid etching[1,2] and followed by extraction of the resins[4] via water-filled nanometer-sized voids within the hybrid layer. The third stage involves the enzymatic attack of the exposed collagen fibrils by endogenous matrix metalloproteinases (MMPs) and cysteine cathepsins.[5,6]

The MMPs are a large family of zinc- and calcium-dependent endopeptidases presented in the human dentin[6-8] that are capable of degrading all extracellular matrix components.[9] By suppressing the collagenolytic and gelatinolytic activities of dentin, one can reduce the degradation of the collagen fibrils,[10] producing bonded interfaces less prone to degradation over time. In fact, previous studies demonstrated that the pretreatment of acid-etched dentin with chlorhexidine digluconate,[11-14] the most tested MMP inhibitor, yielded stable bond strength values after aging.

Several other MMP inhibitors are described in the literature,[15,16] such as ethylene diamine tetra-acetic acid[17,18], tetracycline and chemical-modified tetracyclines (CMTs),[18-21] galardin,[22] polyvinilphosphonic acid,[23] quaternary ammonium compounds,[24,25] green tea polyphenols,[26] and carbodiimides,[27] among others. However, few attempts were made to investigate their effect on prolonging the lifetime of dentin-bonded interfaces.

Recently, Osorio and others[21] and Toledano and others[18] reported that doxycycline, a CMT, was capable of inhibiting collagen degradation in demineralized dentin in periods ranging from 24 hours up to 3 weeks. In another laboratory investigation, the authors reported that the application of 2% minocycline, which is also a CMT, as dentin pretreatment did not affect the immediate performance of etch-and-rinse adhesives,[28] appearing to be a good priming agent to prevent the degradation of the resin-dentin interfaces. More recently, Feitosa and others[29] showed that the inclusion of doxycycline in nanotube-modified adhesive can inhibit MMP activity without jeopardizing the degree of conversion or bond strength to dentin. However, as pointed out in a recent review published by Perdigão and others,[16] the use of CMTs and their analogues in dentin bonding as a way to prolong resin-dentin bonds is still scarce in the literature and should be the focus of further investigation because of the potential role of CMTs on MMP inhibition. Therefore, the aim of this study was to evaluate the effect of a 2% aqueous solution of minocycline when compared to a 2% aqueous solution of chlorexidine digluconate as pretreatment of acid-etched dentin on the longevity of resin-dentin bond strength and nanoleakage (NL) of two-step etch-and-rinse adhesives.

METHODS AND MATERIALS

Tooth Preparation and Experimental Design

Thirty extracted, caries-free human third molars were used. The teeth were collected after the patient's informed consent and the local university review board approved this study (protocol #6280/2009). Teeth were disinfected in 0.5% chloramine, stored in distilled water in the refrigerator (4°C), and used on average 2-3 months after extraction.

A flat and superficial dentin surface was exposed on each tooth after wet grinding the occlusal enamel on 180-grit SiC paper. The enamel-free, exposed dentin surfaces were further polished on wet 600-grit silicon-carbide paper for 60 seconds to standardize the smear layer.

After preparation, teeth were divided into six groups (n=5), according to the combination of the main factors adhesive (Adper Single Bond 2 [3M ESPE, St Paul, MN, USA] and Prime & Bond NT [Dentsply De Trey, Konstanz, Germany]) and rewetting solution (2% aqueous solution of minocycline [MO], 2% aqueous solution of chlorhexidine digluconate [CHX], and water). The composition and batch number of the adhesives are described in Table 1.

Restorative Procedures and Specimen Preparation

The dentin surfaces were conditioned with 37% phosphoric acid gel (Condac 37, FGM, Joinville, Brazil) for 15 seconds, water rinsed for 15 seconds, and air-dried for 30 seconds. In the control group,

TABLE 1: *Adhesive Systems: Composition, Groups and Application Mode*

Adhesive Systems	Composition	Rewetting Solution	Application Mode
Prime & Bond NT	Caulk Tooth Conditioner Gel 34% phosphoric acid Adhesive: UDMA, PENTA, R 5-62-1 resin, T resin, D resin, silanated colloidal silica, cetylamine hydroxyfluoride, initiator, stabilizer, and acetone	Water 2% minocycline 2% chlorhexidine	a: acid etch (15 s); b: rinse (15 s); c: air-dry (30 s); d: rewetting (15 s); e: one coat of adhesive; f: air-dry for 10 s at 20 cm; g: repeat e and f; h: light cure (10 s, 600 mW/cm^2).
Adper Single Bond 2	Scotchbond Etchant 35% phosphoric acid Adhesive: Bis-GMA, HEMA, dimethacrylates, nanofilled colloidal silica (5 nm) polyalkenoic acid copolymer, initiators, water, and ethanol	Water 2% minocycline 2% chlorhexidine	a: acid etch (15 s); b: rinse (15 s); c: air-dry (30 s); d: rewetting (15 s); e: one coat of adhesive; f: air-dry for 10 s at 20 cm; g: repeat e and f; h: light cure (10 s, 600 mW/cm^2).

Abbreviations: UDMA – urethane dimethacrylate; PENTA – dipentaerythritol pentacrylate monophosphate; Bis-GMA: bisphenol A diglycidyl methacrylate; HEMA: 2-hydroxyethyl methacrylate

the dentin surfaces were rewetted with water for 15 seconds, while in the experimental groups, dentin surfaces were rewetted with 2 wt% aqueous solution of MO (Fleming Drugstore, Ponta Grossa, Brazil) or 2 wt% aqueous solution of CHX (FGM) for 15 seconds. All substances were used within 1 week after preparation.

The excess solution was removed with a blotting paper. The adhesive systems were applied according to the manufacturer's instructions (Table 1) and light cured with a quartz-tungsten-halogen light (VIP, Bisco, Schaumburg, IL, USA; 600 mW/cm^2) for 10 seconds. Resin composite buildups (Opallis, FGM) were constructed in three increments of approximately 1.5 mm and individually light activated for 40 seconds. All bonding procedures were carried out by a single operator at 24°C and 50% relative humidity.

The bonded teeth were stored in distilled water at 37°C for 24 hours. After storage, specimens were longitudinally sectioned in both "x" and "y" directions across the bonded interface with a diamond saw (Extec Corp., Enfield, CT, USA) at 300 rpm to obtain bonded sticks with a cross-sectional area of approximately 0.8 mm^2. The number of premature failures during specimen preparation was recorded.

The cross-sectional area of each stick was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm and recorded for subsequent calculation of the microtensile bond strength (μTBS). The bonded sticks that originated from the same tooth were randomly divided for immediate or 24-month testing. The specimens for the 24-month group were stored at 37°C in hermetically sealed vials containing distilled water.

Two resin-bonded sticks, from each tooth and at each storage period, were used for NL evaluation for each experimental condition, and the remaining bonded sticks were tested for microtensile bond strength.

(μTBS) Microtensile Bond Strength Test

Resin-dentin bonded sticks were attached to a μTBS jig (Odeme Prod. Odont. Ltda, Joaçaba, Brazil) with cyanoacrylate adhesive and tested under tension (Emic, São José dos Pinhais, Brazil) at 0.5 mm/min until failure. The μTBS values were calculated by dividing the load at failure by the cross-sectional bonding area.

The failure modes were evaluated at 400× (HMV-2, Shimadzu, Tokyo, Japan) and classified as cohesive (failure exclusive within dentin or composite [C]), adhesive (failure at resin-dentin interface [A]), or adhesive/mixed (failure at resin-dentin interface that included cohesive failure of the neighboring substrates [A/M]).

Nanoleakage (NL)

Two resin-bonded sticks, from each tooth and at each storage period, were used for NL evaluation for each experimental condition (n=10). Ammoniacal silver nitrate was prepared according to the protocol previously described by Tay and others.[30] The sticks were placed in the ammoniacal silver nitrate in darkness for 24 hours, rinsed thoroughly in distilled water, and immersed in photo-developing solution for 8 hours under a fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface. Specimens were polished with a wet 600-, 1000-, 1200-, 1500-, 2000-, and 2500-grit SiC paper and 0.25 μm diamond paste (Buehler Ltd, Lake Bluff, IL, USA) using a polishing cloth.

They were ultrasonically cleaned, air-dried, and acid etched for 3 seconds in a 50% phosphoric acid

TABLE 2: *Number and Percentage of Specimens (%) According to Fracture Pattern in Each Experimental Condition as Well as Total Number of Specimens Tested (TN)*

Rewetting Solution	Prime & Bond NT								Adper Single Bond 2							
	Immediate				24-Mo				Immediate				24-Mo			
	A/M	C	PF	TN	A/M	C	PF	TN	A/M	C	PF	TN	A/M	C	PF	TN
Control	36 (80)	0 (0)	9 (20)	47	45 (78)	1 (2)	12 (20)	58	39 (93)	0 (0)	3 (7)	41	41 (95)	0 (0)	2 (5)	43
2% minocycline	34 (97)	0 (0)	1 (3)	35	33 (94)	2 (6)	0 (0)	35	45 (98)	0 (0)	1 (2)	45	43 (96)	2 (4)	0 (0)	45
2% chlorhexidine	50 (85)	2 (3)	7 (12)	59	43 (94)	1 (2)	2 (4)	46	41 (93)	2 (5)	1 (2)	44	38 (90)	2 (5)	2 (5)	42

Abbreviations: A/M, adhesive/mixed fracture mode; C, cohesive fracture mode; PF, premature failure.

solution followed by immersion in 2% NaOCl for 10 minutes. Specimens were mounted on aluminum stubs and sputter coated with gold (Sputter Coater IC 50, Shimadzu, Tokyo, Japan). Resin-dentin interfaces were analyzed in a scanning electron microscope operated in secondary electron mode (SSX-550, Shimadzu, Tokyo, Japan) at a working distance of 20 mm and 15 kV.

The percentage of NL in the adhesive and hybrid layers of each bonded stick was measured using energy-dispersive X-ray spectrometry (SSX-550, Shimadzu). This measurement was performed in three random regions (5×5 μm) of the bonded stick (left, center, and right). The total area of the hybrid layer–scanned NL measurement was approximately 75 μm^2. The NL was expressed as a percentage of the total area evaluated.[31]

Statistical Analysis

The mean μTBS and the percentage of NL of all bonded sticks from the same tooth were averaged for statistical purposes. Premature failures were included in the tooth mean with an attributed value of 3.4 MPa. This value corresponds to approximately half of the minimum μTBS value that could be measured in this study, which was 6.9 MPa. This approach avoids overestimation of the bond strength means, and it was previously performed in other studies.[32-34]

The μTBS (MPa) and NL (%) data of each adhesive system were subjected to two-way repeated measures analysis of variance (rewetting solution vs storage time) and Tukey test with a level of significance of 5%.

RESULTS

Approximately 18-25 sticks were obtained per tooth, including those with premature failure. The mean cross-sectional area ranged from 0.81 to 0.96 mm^2, and no difference among groups was detected ($p>0.05$).

The percentage of specimens that suffered premature failure and the frequency of each fracture mode are shown in Table 2. Most of the specimens showed adhesive/mixed fractures in all groups.

μTBS Test

The total number of resin-bonded sticks evaluated in the μTBS test is summarized in the Table 2. For both adhesives tested, the cross-product interaction of rewetting solution vs storage time was statistically significant (Table 3; $p=0.001$). Significant reductions of μTBS values after 24 months were observed only for the control groups ($p<0.05$). The dentin pretreatment with MO and CHX produced stable bond strengths after 24 months of water storage (Table 3).

NL

The cross-product interaction of rewetting solution vs storage time was significant (Table 4; $p>0.001$) for both adhesives. For Prime & Bond NT, lower NL was observed in the immediate period for all rewetting substances. After 24 months, the control group

TABLE 3: *Means and Standard Deviations of the Microtensile Bond Strength Values (MPa) for Each Experimental Condition*[a]

Rewetting Solutions	Prime & Bond NT		Adper Single Bond 2	
	Immediate	24-Mo	Immediate	24-Mo
Control	42.3 ± 3.4 A	23.6 ± 5.3 B	46.2 ± 4.7 a	32.3 ± 4.5 b
2% minocycline	46.3 ± 5.4 A	41.4 ± 3.6 A	49.6 ± 3.6 a	44.2 ± 5.1 a
2% chlorhexidine	44.2 ± 4.3 A	36.3 ± 5.1 A	50.3 ± 5.6 a	43.3 ± 3.5 a

[a] *Groups identified with the same upper- or lowercase letters are statistically similar (Tukey test, p>0.05).*

TABLE 4: *Means and Standard Deviations (%) of Nanoleakage at the Adhesive Interfaces for Each Experimental Condition*[a]

Rewetting Solutions	Prime & Bond NT		Adper Single Bond 2	
	Immediate	24-Mo	Immediate	24-Mo
Control	26.2 ± 3.6 BC	51.3 ± 4.1 D	27.9 ± 5.8 b	39.3 ± 4.2 c
2% minocycline	21.2 ± 3.2 AB	28.1 ± 4.5 C	17.2 ± 5.1 a	24.3 ± 3.9 ab
2% chlorhexidine	17.5 ± 4.3 A	32.3 ± 3.9 C	16.2 ± 4.1 a	26.5 ± 3.4 b

[a] *Groups with the same upper- or lowercase letters are not significantly different (Tukey test, p>0.05).*

showed the highest NL compared with MO and CHX groups (Table 4; $p>0.001$). For Adper Single Bond 2, lower NL was observed in the MO and CHX groups at the immediate period. After 24 months of storage time, the control group showed the highest NL compared with the MO and CHX groups (Table 4; $p>0.001$).

Representative scanning electron microscopic images at the resin-dentin interfaces are shown in Figures 1 and 2. Nanoleakage occurred in all experimental conditions, but the highest NL values were observed in the control group after 24 months of storage (Figures 1D and 2D).

DISCUSSION

As already reported in the literature,[13,14] resin-dentin interfaces from the control groups suffered degradation after long-term water storage. Compared with the immediate bond strength values, the present study detected reductions in the range of 39%-50% for both etch-and-rinse adhesives. On the other hand, the addition of a preliminary treatment of the demineralized dentin with MO or CHX produced stable μTBS and reduced NL after 24 months of water storage in comparison with the control group.

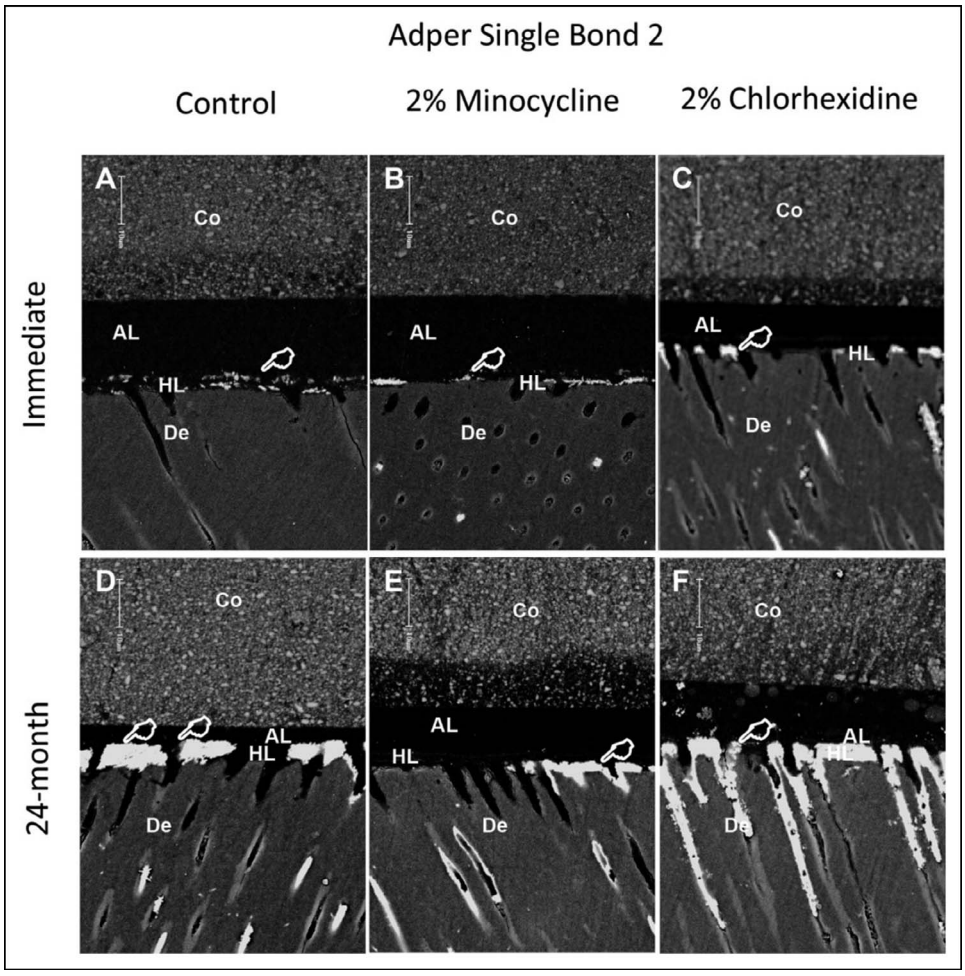

Figure 1. *Representative backscatter SEM images of the resin-dentin interfaces bonded with Adper Single Bond 2. In the immediate time, the amount of silver penetration was lower and practically occurred within the HL (A-C). Few dentin tubules were infiltrated by silver nitrate for all groups (arrows in A-C). After 24 months of water storage, the amount of silver nitrate increased, with deposition of silver deposits occurring throughout the entire thickness of the HL and part of the AL, mainly in the control group (arrows in D) (SEM, scanning electron microscopy; Co, composite; De, dentin; HL, hybrid layer; AL, adhesive layer).*

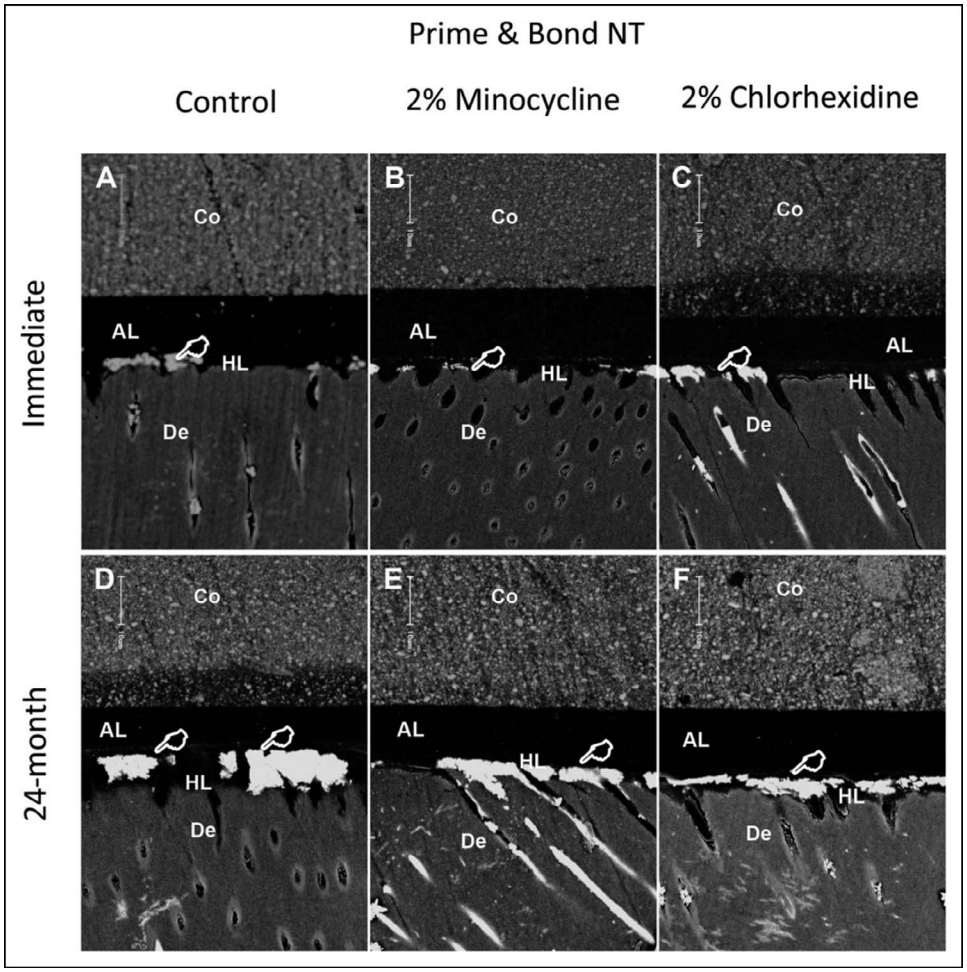

Figure 2. *Representative backscatter SEM images of the resin-dentin interfaces bonded with Prime & Bond NT. Lower percentage of silver penetration was shown in the immediate groups (A-C), mainly in the HL (arrows). In all groups, an increased amount of silver nitrate after 24 months of water storage occurred (D-E). This increase was significantly higher in the control group, with most silver uptake deposits occurring throughout the entire thickness of the HL and part of the AL (arrows in D) (SEM, scanning electron microscopy; Co, composite; De, dentin; HL, hybrid layer; AL, adhesive layer).*

The protective effect of 2% CHX was not surprising. The CHX molecule is characterized by being a strong base with cationic properties when ionized in a solvent such as water, and this cationic part of the molecule connects to the negatively charged part of the collagen or MMPs. It is possible that CHX binds to phosphate groups or to negative carboxyl groups in mineralized dentin crystallites or collagen matrix, respectively,[35] and due to its substantivity can remain bonded in demineralized and mineralized dentin substrates.[36]

CHX was extensively evaluated as dentin pretreatment to prolong the lifetime of bonded interfaces.[37,38] More recently, its incorporation into acidic conditioners and some adhesive systems also produced stable bond strengths after water storage.[14,39-42] The inhibition of MMP-2 and MMP-9 has been attributed to the chelating ability of CHX to zinc.[43] In the case of MMP-8, CHX interacts with the essential sulfhydryl groups and/or cysteine of the active enzyme sites. Additionally, recent investigations reported that CHX could also inhibit the activity of cysteine cathepsins,[5] which constitute another dentin protease likely involved in the degradation of the bonded interfaces.[6]

Similarly to CHX, the chemically modified tetracycline (CMT) tested also produced stable bond strengths after 24 months of water degradation. Apart from the lack of antibiotic activity, CMTs such as minocycline are potent MMP inhibitors.[44] Although the effect of minocycline on dentin MMPs was not investigated, doxycycline, which is another CMT, was shown to be even more effective than CHX in inhibiting MMPs activity. While CHX exerted a partial, time-limited MMP inhibition, minimizing collagen degradation by 57% for only 24 hours, doxycycline fully blocked proteolysis for periods of up to 3-4 weeks.[18,21]

It is fair to hypothesize that both CMTs share similar MMP inhibition potential due to their comparable chemical structure.[19] Doxycycline and minocycline show a basic chemical structure consisting of a tetracyclic naphthacene carboxamide ring

system. These structures differ by doxycycline having one hydroxyl group in carbon 5 of ring B and one methyl group in carbon 6 of ring C. In contrast, minocycline has one amine group on carbon 7 of ring D.[44] Despite these differences, both substances can inhibit collagenases and gelatinases.[45,46] In the present study, we did not investigate the effect of doxycycline on the durability of the resin-dentin bonds since an earlier study reported that doxycycline, when mixed with adhesive solutions, resulted in phase separation and lowered the immediate bond strength of etch-and-rinse adhesives to dentin,[28] and this is probably the reason for recent attempts to encapsulate doxycycline.[29]

CMTs are considered broad-spectrum MMP inhibitors.[19,43] Their inhibitory effects were attributed to their ability to chelate Ca^{+2} and Zn^{+2},[46,47] which are two essential ions for MMPs to maintain their structure and functional active sites.[9] Zinc is bound to the catalytic domain of the enzyme, and calcium is required to produce MMP activation.[18,48] Therefore, by binding to Zn and Ca, CMTs inactivate the endogenous proteases. However, the hypothesis that binding of zinc results in protection of sensitive cleavage sites of metalloproteinases requires further validation since zinc in excess reduces MMP-mediated collagen degradation.[21,49]

Another likely explanation for the MMP inhibition produced by CMT is that it acts by altering MMP conformation rather than by interaction with the catalytic zinc.[18,48] This would explain its long-lasting and more potent effect when compared to CHX.[18,21]

Another important finding of the present investigation was the reduced nanoleakage of the experimental controls after 24 months. The nanoleakage reveals the location of defects at the resin-dentin interface that could work as pathways for degradation of resin-dentin bonds over time. Silver nitrate occupies nanometer-sized spaces around naked collagen fibrils, where resin failed to infiltrate or where residual water was not displaced by the adhesive resin.[50]

This seems to worsen during aging, as naked collagen fibrils are digested by the endogenous proteases, increasing the size and volume of defects in the hybrid layer. In the face of that, we hypothesize that the reduced nanoleakage of the MO and CHX groups after 24 months compared to the control groups shows that less degradation of the collagen fibrils occurred during this water storage period—more indirect evidence of the MMP potential of both MO and CHX.

In spite of the promising results obtained with minocycline in the present study, some authors report that their use in association with dentin bonding systems is limited since they may stain teeth with a purple hue after photo-oxidation of the CMTs.[2,51] A similar concern was already raised for CHX,[51,52] as it can also stain teeth.

Although these concerns are factual, they were never observed under the protocol herein investigated in clinical studies.[53-55] In the present investigation, none of the specimens showed color change over the course of these 24 months, suggesting that this may not be a problem for MO and CHX when used as dentin priming conditioners. Under this protocol, the products were applied only once for a short duration and with a reduced volume of the product. However, further investigations should be conducted to rule out completely this potential side effect of CMTs on dentin bonding.

CONCLUSION

Within the limits of this study, it was found that the use of 2% minocycline as a dentin priming solution after acid etching is an alternative to retarding the degradation of resin-dentin interfaces over a 24-month period.

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Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial or other personal interest of any nature or kind in any product, service or company that is presented in this article.

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