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EVALUATION OF MATRIX METALLOPROTEINASES BIODEGRADATION INHIBITION PROTOCOLS ON RESIN-DENTINE INTERFACIAL CHARACTERISTICS (AN IN VITRO COMPARATIVE STUDY)

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ABSTRACT

Objective: to evaluate and compare the effect of different matrix metalloproteinases biodegradation inhibition protocols. **Material and methods:** 192 permanent molar sound teeth were collected. Using a diamond disk, wet grinding of the occlusal surface of all samples into flat surface to expose the superficial dentinal surface. 37% phosphoric acid was applied for 15 seconds, cleaned with water and dried with cotton rolls. Then, the molar teeth were divided into four main groups (n=48); group I; No treatment (control group), group II; Chlorhexidine (2%); group III; Minocycline (2%); group IV; Sodium Hypochlorite (5.25%). Samples were cleansed with distilled water and the adhesive bonding of the dentine surface was performed and restored with light cure nanohybrid resin composite. Samples were longitudinally sectioned perpendicular to the adhesive interfaces and prepared according to test before and after thermocycling. **Results:** For microtensile bond test, control group and minocycline showed highest significant microtensile bond strength in comparison with chlorohexidine and sodium hypochlorite immediately and after thermocycling. While no significant difference was found between different groups for failure mode analysis. For nanoleakage, control group and minocycline showed the least nanoleakage. For hybrid layer resin tag length evaluation control group, minocycline and chlorohexidine groups showed almost similar resin tag penetration while sodium hypochlorite showed the highest penetration for resin tags. **Conclusions:** Usage of minocycline matrix metalloproteinase inhibitor showed promising results concerning bond strength and nanoleakage on contrary to chlorohexidine. Sodium hypochlorite results showed decreased bond strength, increased nanoleakage and resin tag length.

KEYWORDS: Matrix metalloproteinase enzymes, MMPs inhibitors, deproteinizing agent, microtensile strength test and Nanoleakage.

INTRODUCTION

Matrix Metalloproteinase enzymes (MMPs); has been proposed as a regulatory mechanism necessary for correct mineralization of the dental structure which were involved in the process of reabsorption of extracellular matrix proteins[(1)]. Some are trapped inside the mineralized dentine matrix during dentinal development in the form of proenzymes, they can hydrolyze the components of

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the extracellular matrix when activated by physical or chemical stimuli because of their collagenolytic protease activity[2]. The process of activation is initiated by the breakdown of the zinc-cysteine interaction, which is called the cysteine switch. This is the key to understanding the function of these enzymes and means of their inhibition protocols[3].

The use of MMPs inhibitors has been introduced to dentistry to stop action of those enzymes and increase bonding strength of bonding agent to Dentine hybrid layer (DHL). Such as the use of Chlorhexidine (CHX) and Tetracycline compounds as Minocycline (MI), but CHX was proven to be water soluble and lack the ability to bind to demineralized dentine matrix which could limit its use in dental adhesives as it may diffuse out of the matrix as it is unable to copolymerize and probably would leach out over time[4]. While MI has received increased interest as they compete with MMPs for calcium and zinc ions as metal chelating agents to inhibit their activity, this slows collagen degradation in the DHL and improves bonding strength[5]. Tetracycline compounds can also prevent collagen degradation and improve stability in the DHL.

Hence, this study was formulated to evaluate the effect of different MMPs inhibitors and their effect on DHL integrity together with microtensile test, failure pattern of resin dentine interface and nanoleakage all before and after thermocycling.

MATERIAL AND METHODS

The materials used in this study were, Nano-hybrid resin composite (Resin Filtek Z250 XT, 3M, USA) and solobond M adhesive bond (single etch, VOCO, Germany), 2% Minocycline (Penta pharm, Egypt) and 2% chlorhexidine (Dental plus co., Egypt), 5.25% Sodium Hypochlorite (Dental plus co., Egypt), 37% Meta etchant (BIOMED, Korea).

Grouping of samples:

A total of 192 mandibular molar teeth were used in this study and divided into four equal main groups (n=48) according to the biodegradation inhibition treatment protocols as follow:

- Group I: No treatment, only acid-etching (control group).
- Group II: Protease inhibitor with 2% chlorhexidine for 5 minutes[6].
- Group III: Protease inhibitor with 2% minocycline for 5 minutes[6].
- Group IV: Deproteinized with 5.25% sodium hypochlorite for 1 minute[7].

Afterwards each group was subdivided into three equals (n=16) according to the test type; hybrid layer examination, nanoleakage, and microtensile bond strength followed by failure mode analysis.

Teeth preparation:

Freshly extracted permanent mandibular molar teeth free from cracks (tested by using transillumination), caries or restorations were collected and used in this in vitro study. Using a diamond disk wet grinding of the occlusal surface of all the samples were into a flat surface to expose the superficial dentinal surface. 37% phosphoric acid was applied for 15 seconds, washed with water and dried with cotton rolls[6]. Samples groups were treated by different protocols previously mentioned then the samples were cleansed with distilled water, the adhesive bonding of the dentine surface was performed, after that the samples were restored with light cure nanohybrid resin composite[7].

Each group was subdivided into three equals (n=16) according to the type of test, half of each subgroup were tested immediately, the other half was tested after thermocycling (THE- 100 SD thermocycler, Mechatronic, Germany) for 2500 cycles between cold water 5°C for 30 seconds then 10 seconds of rest then hot water 55°C for 30 seconds with a dwell time of 30 seconds to simulate intraoral biodegradation process then the samples were sectioned according to each test[6].

Evaluation of resin-dentine interface and resin tags

Bonded specimens of each group were stored in 37°C distilled water for 24 h before sectioning. Sectioning of teeth into 1mm slabs longitudinally with isomet (Isomet 4000, Buehler, USA) perpendicular to the adhesive interfaces were done and polished with #600, 1200, 1500, 2000, 2500 and 3000 silicon carbide papers for 30 s under cooling and polished with diamond paste. Specimens were sputtered with gold for scanning electron microscopy (SEM) examination. SEM was used to examine the hybrid layer of teeth samples to evaluate the depth of resin penetration, before and after thermocycling degradation[8].

Evaluation of nanoleakage at Dentine hybrid layer

Samples were sectioned into 1 mm thick slabs using isomet perpendicular to adhesive interface to investigate nanoleakage. The bonded slabs (16/ group) were coated with two layers of nail varnish applied up to 1 mm from the bonded interface, then immersed in an 50% ammoniacal silver nitrate solution in total darkness for 24 h and prepared according to the protocol previously described by Tay et al.[9]. They were then removed, properly rinsed under running tap water and immersed in photo developing solution for 8 h under a fluorescent light to reduce the penetrated silver ions into metallic silver grains[9].

To remove the superficial silver adsorption, specimens were then gently polished with 600, 800, 1200, 1500, and 2000 grit silicon carbide paper and placed in an ultrasonic cleaner for 5 min.

Then prepared slabs of each group were divided into half, one to be tested without thermocycling and other after thermocycling, immersion in silver nitrate was done after thermocycling for subgroups to be thermocycled. Examination was done for prepared slabs at 1000× magnification with scanning electron microscope[10]. Analyzing the distribution of metallic silver particles at the dentine-resin interface was done using the digital image software Fiji (https://imagej.net/Fiji) [11], in a selected area (height × width = 21 × 250 μm) on each image. The Otsu method was used for the SEM images to convert into binary images; thus, the silver area was identified as black pixels on a white background, and the percentage of silver penetration at the interfacial zone was calculated[10,12].

Evaluation of microtensile bond strength:

I. Beam Preparation:

Restored teeth were sectioned longitudinally to obtain resin-dentine beams of 1 mm×1 mm. In order to facilitate identification of beam location in restored teeth whether peripheral or central, the surfaces of composite restorations were painted with permanent ink so that the end of central beams would have a different color from peripheral ones[9]. A gripping attachment was designed specially to hold acrylic blocks firmly with mounted teeth parallel to the sectioning direction, so the relation between the cutting disc and the occlusal surface was maintained perpendicular. The L-shaped attachment is composed of a cylindrical metal ring (16-mm in diameter, 3-mm height, 2-m thickness) soldered at its base to a metal rod, which is used to mount the attachment into the diamond saw machine. Two axial grooves, perpendicular to each other, were made on top surface of metal ring to facilitate accurate positioning and rotation of acrylic blocks inside the gripping attachment[12].

After mounting blocks in the gripping attachment, restored teeth were serially sectioned at 2050 rpm under copious coolant. Serial sectioning was done in Bucco-lingual direction then rotated 90° clockwise and sectioned in Mesio-distal, a final horizontal cut at level of Cemento-enamel junction was done to obtain beams. Resultant beams were 0.9±0.1 mm in thickness and 5.5±1 mm in length and beams were stored in a tight-seal plastic cone labeled according to subgroup and tooth of origin in distilled water at room temperature. After 24h, half of the specimens (n=8 teeth) were thermocycled as mentioned before[9].

II. Micro-tensile bond strength measurement:

For each tested subgroup, 16 beams were tested. Geraldeli's jig was used to mount beams onto the universal testing machine (Instron universal testing machine model 3345 England). Then the jig was mounted into the universal testing machine with a load cell of 500 N[9].

Tensile load was applied, at a crosshead speed of 0.5 mm/min, until bonding failure of the specimen occurred. Bond strength was calculated in MPa (data calculated and recorded using computer software bluehill Instron England). A scalpel was used to carefully remove specimen fragments from the jig and stored in their labelled plastic cones until failure mode examination[12].

Evaluation of failure pattern:

Failure modes was evaluated by stereomicroscopy (Nikon MA 100 stereomicroscope Japan 50x magnification with Omnimet Buehler Germany image analysis software), and type of failure either cohesive in dentine (CD: fracture within dentine substrate only), cohesive failure in composite (CC: fracture within composite substrate only), adhesive (A: fracture between dentine and composite interface) or mixed failure (M: any of 2 previous failure pattern). After testing, the fracture modes were evaluated and classified according to the predominant mode of fracture[13].

Statistical analysis

Data presented as mean, standard deviation (SD), Median, Minimum, and Maximum when appropriate. Data explored for normality using Shapiro-Wilk test. Microtensile bond strength (μTBS) and nanoleakage showed normal distribution, so Two-way ANOVA used to compare between tested groups and thermocycled, followed by Tukey HSD for pairwise comparison. Resin tag length and failure mode analysis showed non-normal distribution, so to compare between tested groups Kruskal Wallis test was used, while to compare between before and after thermocycling Mann Whitney test was used. The significance level was set at $p < 0.05$. Statistical analysis was performed with IBM® SPSS® (ver. 26. SPSS Inc., IBM Corporation, Armonk, NY, USA).

RESULTS

Hybrid layer evaluation and resin tag length (table 1):

Before thermocycling (No TC); 5.25% NaOCl group (31.3±8.79) showed higher significant resin tag length compared to control group (13.42±6.26), 2% Chlorhexidine group (17.68±5.6), and 2% Minocycline group (16.15±3.17) at p<.001.

After thermocycling (TC); control group (12.47±6.34) showed higher significant resin tag length compared to 2% Chlorhexidine group (21.31±5.57), 2% Minocycline group (20.92±6.05), and 5.25% NaOCl group (24.53±6.48) at p<.001.

TABLE (1) Mean and Standard deviation (SD) results of resin tag length for thermocycling for different groups.

	Control		2% Chlorhexidine		2% Minocycline		5.25% NaOCl		p-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
No TC	13.42[b]	6.26	17.68[b]	5.6	16.15[b]	3.17	31.3[a]	8.79	<.001*
TC	12.47[b]	6.34	21.31[a]	5.57	20.92[a]	6.05	24.53[a]	6.48	<.001*

Significant difference is indicated by different letters within each row at p<.05

**=significant, NS=non-significant*

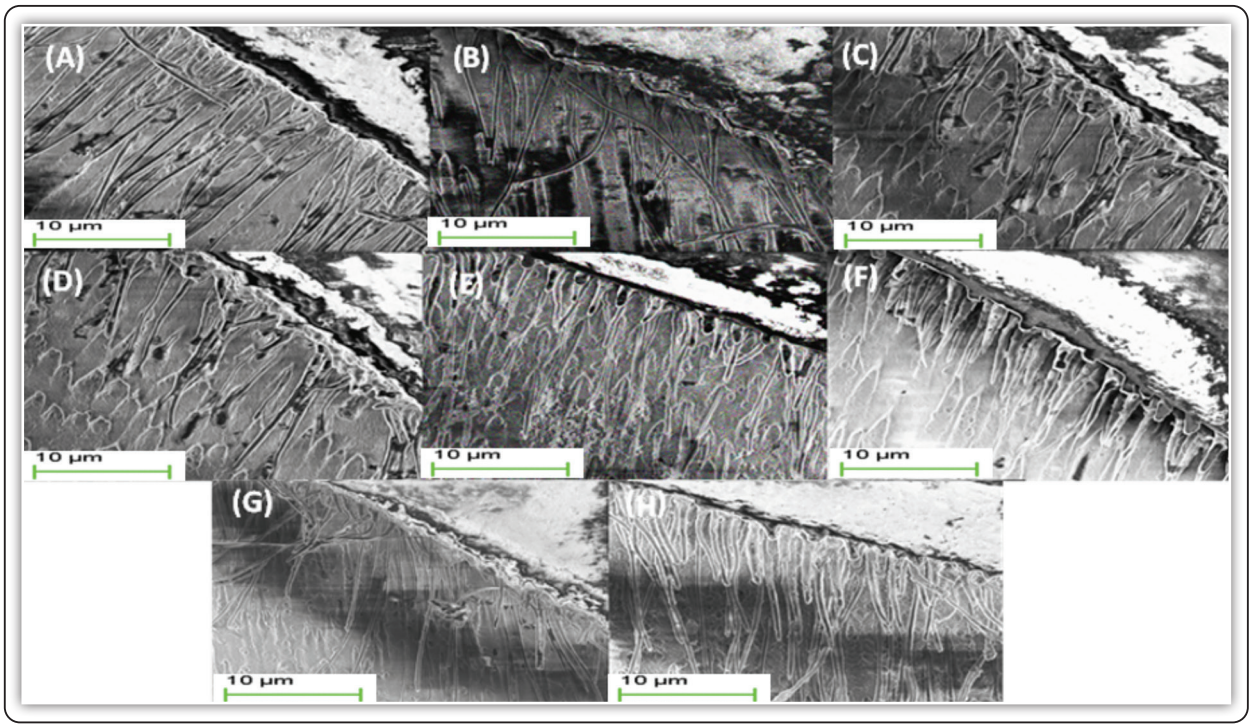

FIG (1) Showing SEM images showing (A, C, E, G) resin tags in control, minocycline, chlorhexidine and sodium hypochlorite groups respectively before thermocycling and (B, D, F, H) resin tags in control, minocycline, chlorhexidine and sodium hypochlorite groups respectively after thermocycling.

Nanoleakage test results (table 2):

Before thermocycling (No TC); control group (0.53±0.24) showed lower significant nanoleakage compared to and 2% Chlorhexidine group (4.93±0.95) and 5.25% NaOCl group (4.02±0.65) at p=.007, while 2% Minocycline group (2.05±1.29) showed insignificant difference compared to control and NaOCl. After thermocycling (TC); control group (1.01±1.03) showed lower significant nanoleakage compared to and 2% Chlorhexidine group (9.97±2.14) and 5.25% NaOCl group (8.78±4.07) at p=.001, while 2% Minocycline group (5.72±2.55) showed insignificant difference compared to all other groups.

TABLE (2) Mean and Standard deviation (SD) results of nanoleakage for thermocycling for different groups.

	Control		2% Chlorhexidine		2% Minocycline		5.25% NaOCl		p-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
No TC	0.53^{c}	0.24	4.93^{a}	0.95	2.05^{bc}	1.29	4.02^{ab}	0.65	0.007*
TC	1.01^{b}	1.03	9.97^{a}	2.14	5.72^{ab}	2.55	8.78^{a}	4.07	0.001*

Significant difference is indicated by different letters within each row at p<.05

**=significant, NS=non-significant*

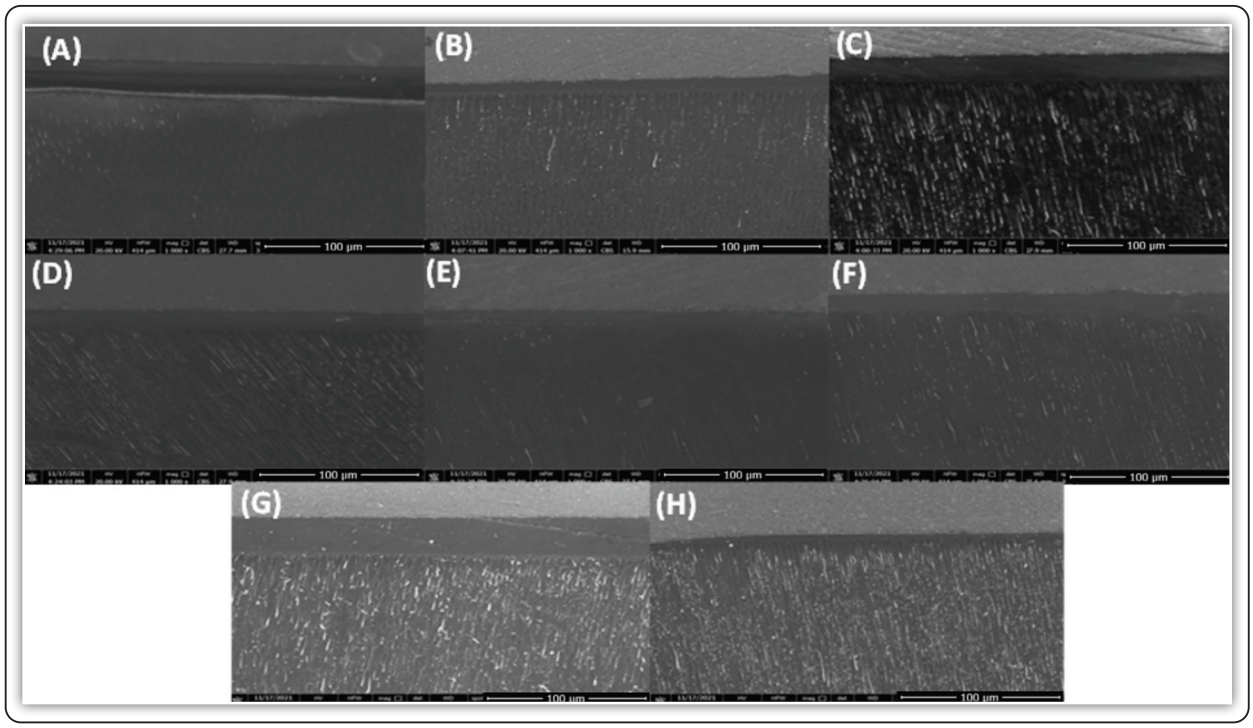

FIG (2) Showing SEM image showing nanoleakage (A, B, C, D) control, minocycline, chlorhexidine and sodium hypochlorite groups respectively before thermocycling (D) sodium hypochlorite group before thermocycling (E, F, G, H) control, minocycline, chlorhexidine and sodium hypochlorite groups respectively after thermocycling.

Microtensile bond strength (μTBS) (table 3):

Before thermocycling (No TC); control group (39.55±8.98) and 2% Minocycline group (36.15±12.25) showed higher significant μTBS compared to 2% Chlorhexidine group (25.48±8.07) and 5.25% NaOCl group (23.67±10.09) at p<.001. After thermocycling (TC); 2% Minocycline group (30.98±9.94) showed higher significant μTBS compared to 2% Chlorhexidine group (20.77±6.67) and 5.25% NaOCl group (20.84±6.04) at p=.003. Control group (27.13±10.35) showed an insignificant difference with all tested groups at p>.05.

Failure mode analysis (table 4):

Insignificant difference resulted between tested groups at p=0.108 before thermocycling and insignificant difference resulted between tested groups at p=0.193 after Thermocycling.

TABLE (3) Mean and Standard deviation (SD) results of μTBS for thermocycling for different groups.

	Control		2% Chlorhexidine		2% Minocycline		5.25% NaOCl		p-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
No TC	39.55[a]	8.98	25.48[b]	8.07	36.15[a]	12.25	23.67[b]	10.09	<.001*
TC	27.13[ab]	10.35	20.77[b]	6.67	30.98[a]	9.94	20.84[b]	6.04	0.003*

Significant difference is indicated by different letters within each row at p<.05

**=significant, NS=non-significant*

TABLE 2: Failure mode distribution for the effect of thermocycling within each group.

		CD	CC	A	M	p-value
No TC	Control	19.05%	0.00%	9.52%	28.57%	0.108 NS
	2% Chlorhexidine	19.35%	3.23%	16.13%	16.13%	
	2% Minocycline	19.23%	7.69%	19.23%	0.00%	
	5.25% NaOCl	15.38%	7.69%	11.54%	23.08%	
TC	Control	9.52%	9.52%	14.29%	9.52%	0.193 NS
	2% Chlorhexidine	16.13%	6.45%	22.58%	0.00%	
	2% Minocycline	26.92%	11.54%	15.38%	0.00%	
	5.25% NaOCl	15.38%	0.00%	23.08%	3.85%	

**=significant, NS=non-significant*

Tereomicroscopy image showing cohesive composite failure

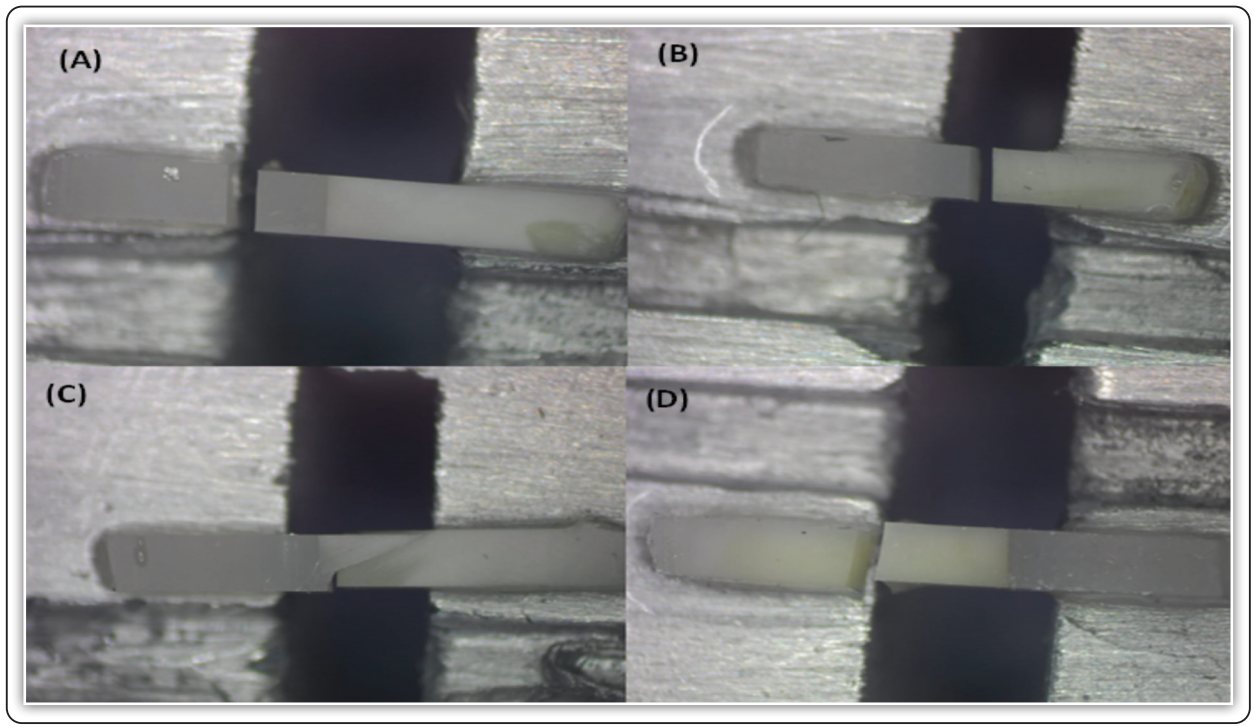

FIG (3) Showing stereomicroscopy image (A) showing cohesive composite failure (B) showing adhesive bond failure (C) showing mixed cohesive adhesive failure (D) showing cohesive dentin failure

DISCUSSION

In the current study thermocycling resulted in decreasing the μTBS for only control group. This may be attributed to fluctuation in temperatures during thermocycling, leading to accumulation of thermal stress at the adhesive interface[14,15]. Additionally, HEMA presented in the total etch adhesive is hydrophilic and more liable for water degradation during water storage and thermocycling[14,15].

On the other hand, groups treated with MMPs inhibitors and NaOCl showed an insignificant decrease in microtensile bond after 2500 thermocycles. Minocycline as an MMPs inhibitor react with two essential ions for MMPs to maintain their structure and functional active sites which are zinc and calcium ions, Zinc is bound to the catalytic domain of the enzyme, and calcium is required to produce MMP activation[16]. Therefore, by binding to Zn and Ca minocycline inactivate the endogenous proteases thus preventing collagen degradation in the DHL and improves bonding strength[7].

Also, the CHX molecule when ionized in a solvent such as water is characterized by being a strong base with cationic properties, and the negatively charged part of the collagen or MMPs is connected to this cationic part of the molecule[7]. It is possible that CHX binds to negative carboxyl groups or to phosphate groups in collagen matrix or mineralized dentine crystallites and due to its substantivity can remain bonded in mineralized and demineralized dentine[3].

On the other hand, sodium hypochlorite solution is a nonspecific proteolytic with antibacterial properties, which can remove the smear layer organic phase of the dentine structure[17]. The proteolytic effect of NaOCl leaves dentine surface with higher mineral content, which has favorable bonding potential rather than exposed collogen fibers[17,18]. Moreover, mineralized dentine presents higher surface energy than the exposed collagen fibrils at dentine surface and substrate free energy might be increased by hypochlorite, improving monomer impregnation and wettability of dentine wettability[19].

It is worth to mention that the collagen network elimination would result in a surface similar to that in etched enamel[20]. Also, dentine surface tension would be decreased by NaOCH, improving the penetration and compatibility to hydrophobic monomers compared to acid etched dentine[21]. The etch-and-rinse technique was used to overcome these shortcomings, aiming to remove the collagen fibrils exposed by the phosphoric acid etchings so dentine deproteinization has been suggested[22,23].

Minocycline resulted in the highest μTBS before and after thermocycling compared to CHX and NaOCl groups. Presumable reason is that MI can bind with enzyme-associated calcium and changes MMPs form, making it susceptible to proteolytic digestion[24]. That may explain the long lasting and potent inhibition effect that is produced by MI by reacting with Ca and Zn rather than by interaction with the catalytic zinc only as with CHX[7]. Regarding NaOCl, the remnants of super-oxide free radicals generated within the dentine surface may inhibit polymerization of resin monomers and significantly reduces the bond strength to dentine[7,25], also this was confirmed by a higher nanoleakage for NaOCl than control group after thermocycling.

The location of defects at the resin-dentine interface was revealed at nanoleakage test that could work as pathways for resin-dentine bonds degradation over time. Around naked collagen fibrils silver nitrate occupies nanometer sized spaces, where residual water was not displaced by the adhesive resin or where resin failed to infiltrate[15]. This seems to worsen during thermocycling, as naked collagen fibrils are digested by the endogenous proteases, increasing the size and volume of defects in the hybrid layer[10,26].

In our results CHX showed significant increase in gap leading to increased nanoleakage after thermocycling and this could be explained by the chlorhexidine pretreatment of etch-and-rinse adhesive bonded to dentine failed to prevent hybrid

layer degradation after thermocycling[8]. Although the application of chlorhexidine with a etch-and rinse technique on etched dentine surfaces prevented the collagen matrices from collapsing, it caused incomplete water removal from the interfibrillar collagen matrix rendered to the chlorhexidine and was dissolved by water that functioned as the desorption medium[18,27].

Effective debinding of chlorhexidine from the dentine matrix based on water electrostatic binding characteristics because water is qualified as the strongest known H-bonding solvent. H-bonds with collagen molecules rather than with chlorhexidine could be done by water, thereby causing CHX debinding and leaching out of the hybrid layer[18,28].

While concerning resin tag length, the resin tags length was increased with all groups except control group, and this may be attributed to using active scrubbing after application of different treatment methods which removes a part of remaining smear layer along with some of the residual material allowing adhesive for further penetration in microtags[15,18]. NaOCl also removes the organic phase of the smear layer due to its deproteinization effect, leading to adhesives enhanced infiltration into the underlying dentine and chemical bonding to mineral components of the dentine[17,25], and this explains that the NaOCl was the highest in length of resin tags of all groups.

CONCLUSIONS

Within limitation of this study the following conclusions could be drawn:

1. Promising results of minocycline matrix metalloproteinase inhibitor showed in this study concerning nanoleakage and bond strength.
2. Using of chlorohexidine showed less results than minocycline especially concerning nanoleakage.
3. Sodium hypochlorite has showed decreased bond strength and increased nanoleakage and increased resin tag length.

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