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EFFECT OF MATRIX METALLOPROTEINASE INHIBITORS ON MICROTENSILE BOND STRENGTH TO DENTIN USING SELF-ETCH ADHESIVE - IN VIVO STUDY

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ABSTRACT

Objective: to investigate the effect of matrix metalloproteinase (MMPs) inhibitors in vivo on micro-tensile bond strength of resin composite to dentin using self-etch adhesive. **Subjects and Methods**: Nine adult mongrel dogs were included in the study. A total of 90 standardized class I cavity were prepared in upper and lower (canine - first and second molar) in dog mouth. The teeth were divided into three main groups (n = 30) according to the type of MMP inhibitors were used: the control group (no MMPs inhibitors were applied), the CHX group (2% chlorhexidine digluconate, Kempetro, A.R.E), and the EDTA group (Ethylene diaminetetra acetic acid, META BIOMED, CO.LTD, KOREA). Each group were divided into two subgroups (n=15) according to the testing periods 6 months, and 12 months. At the end of each testing period, animals were sacrificed, then teeth were separated from the jaws. Each tooth was mounted on the cutting machine, and sectioned into a series of 1mm thick slabs under water cooling. Micro-tensile bond strength was measured for each sample by using universal testing machine. Data were tabulated and statistically analyzed. **Results**: Results of micro-tensile bond strength showed that after 6 months, CHX significantly revealed higher values than EDTA while after 12 months, CHX significantly revealed lower value than EDTA and control groups. **Conclusion:** The application of EDTA improve micro-tensile bond strength after 12 months of aging, while the bond strength decreased by aging for CHX and control groups.

KEYWORDS: Chlorhexidine Digluconate, EDTA, Matrix Metalloproteinase Inhibitors, Microtensile Bond Strength, Self-etch Adhesive.

INTRODUCTION

The goal of restorative dentistry is to achieve a perfect bond between resin composite and tooth substrates [(1)]. Numerous trials were done to decrease microleakage and preserve restoration integrity. The bonding between the tooth substrate and adhesive materials is called the hybrid layer [(2)]. A perfect hybrid layer formation composed of collagen fibrils embedded by methacrylate-based resins has been considered essential to provide durable and successful adhesion to dentin [(1)]. The absence of stability of the hydrophilic resin components that comprise the hybrid layers is directly related to the breakdown of resin-bonded interfaces, leading to incomplete infiltration of the resin to the full depth of the hybrid layer. This was accompanied by the breakdown of the non-capsulated collagen fibrils at the bottom of the hybrid layer [(3)].

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Longevity and durability of the adhesive joint are affected by degradation of collagen fibrils. Activation of the host-embedded enzymes in the dentin matrix (which known as matrix metalloproteinases (MMPs) enzymes) is one of the reasons behind degradation of collagen fibrils[4]. These (MMPs) enzymes have an important role in many physiological and pathological processes taking place in dentin. Degradation of collagen fibrils that are exposed by infiltrating dental adhesive systems after acid etching is one of these processes [5].

Matrix metalloproteinases (MMPs) enzymes are a group of zinc and calcium dependent enzymes produced by odontoblasts and are trapped inside mineralized dentin matrix[4,6]. The pH changes caused by acidity of monomers in dental adhesive or the pH fluctuations due to cariogenic challenges can activate these enzymes [4]. Exposure of collagen fibrils during the acid etching procedures lead to their hydrolytic and enzymatic degradation, this process mediated by activation of dentin (MMPs)[7].

Self-etch adhesives have been proven to release and activate endogenous MMPs during the dentin bonding procedure[5,8]. Moreover, they might lead to the formation of incompletely infiltrated zones and denuded collagen fibrils along the base of the hybrid layer as a result of the decreasing gradient of resin monomer diffusion within the acid-etched dentin[9]. Recently, several approaches have been suggested to curb the activity of these endogenous enzymes utilizing nonspecific protease inhibitors. These products can prevent collagen degradation and disintegration of bonding interface overtime, because they have an inhibitory effect on the MMPs activity in dentin [10].

Chlorhexidine (CHX) is one of the MMP inhibitors, which is cationic, antibacterial, antiseptic, and used in oral health in wide range [11]. It is usually added to an acid to form a water-soluble salt, such as chlorhexidine digluconate. CHX is an amphiphilic molecule that binds to different proteins by a cation-chelation mechanism and may decrease the catalytic activity of MMPs by binding with Zn+2 or Ca+2 [12,13].

Ethylene diaminetetra acetic acid (EDTA) is a mild chelator at normal pH, with different effects on dentin, according to application time and concentration[14]. It removes the smear layer and slightly demineralizes dentin surface. Its molecule has four carboxylic acid groups, that has the ability to chelate calcium [15]. It has been used in a wide range to dissolve the mineral phase of dentin without alteration of dentin proteins, hence avoiding major alterations of the native fibrillar structure to dentine collagen [7]. EDTA inhibits MMP enzymes and its application on dentin is able to inactivate the endogenous MMP action[16].

Improvement of the bond durability by using MMPs inhibitors has been suggested by many authors[9,17,18]. Despite of the studies on enzyme inhibitors are available, their influence on the bond durability of adhesives remains unclear[19]. Due to limited in-vivo studies on the efficacy of (MMPs) inhibitors on dentin bond strength, this in-vivo study aimed to evaluate the effect of matrix metalloproteinase inhibitors on microtensile bond strength to dentin using self-etch adhesive.

MATERIALS AND METHODS

Selection and preparation of animal:

The research was carried out in accordance with the international guiding principles for biomedical research including animals [16]. A total number of 9 adult mongrel dogs with average weight (10-15 kilogram), and age range between (12 -18 months) were used. All dogs were systemically healthy and showed no clinical signs of dental disorders. They were inoculated against hepatitis, canine distemper, rabies and dewormed against internal parasites and subjected to insecticidal dipping for ectoparasites[16].

All operations were done under general anesthesia consisting of premedication with a mixture of atropine sulphate (0.05mg/kg body weight) and diasepam (1mg/kg body weight) intravenously.

Intravenous anesthesia was induced through cannula by injection of a mixture of ketamine (10mg/kg body weight) and xylazine (1mg/kg body weight). The anesthetic depth was maintained with 2.5% thiopental sodium intravenously. This technique provided deep anesthesia, muscle relaxation and smooth delayed recovery [20].

Sample Size Calculation:

Before the study, the number of teeth required in each group was determined after a power calculation based on data obtained from a pilot study. In that study, the micro tensile bond strength in the control group was 27.53±3.22, the CHX group was 23.25±3.17, and the EDTA group was 28.61±4.57. A sample size of 15 teeth in each group was determined to provide 99% power at 0.05 significance using G Power 3.19.2 software Figure (1).

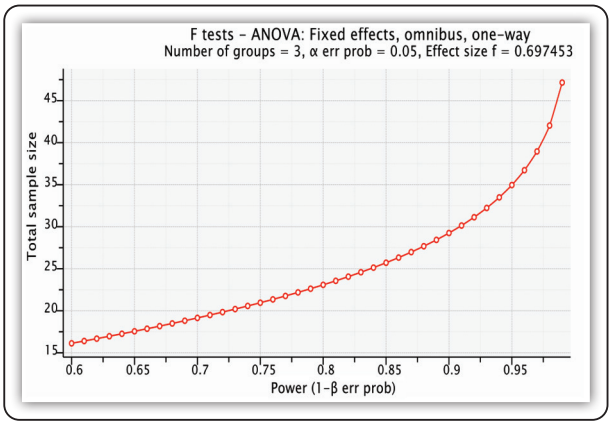

FIG (1) ANOVA test used for sample size calculation.

Experimental study design:

A total number of 90 standardized class V cavities were prepared in the upper and lower (canine, first and second molar) in dog's mouth. Then, the teeth were divided into 3 main groups (n = 30) according to the type of MMP inhibitors were used: the control group (no MMPs inhibitors were applied), CHX group (2% chlorhexidine digluconate, Kempetro, A.R.E), and EDTA group (Ethylene diaminetetra acetic acid, META BIOMED, CO.LTD, KOREA). Each group were divided into 2 subgroups (n=15) according to the testing periods 6 months, and 12 months.

Teeth preparation:

Class V cavity were prepared in the buccal surface of teeth using round carbide bur (#H2, 204, size 014, Komet, USA). The cavities were standardized by measuring (3mm in width and 3 mm in depth) using periodontal probe to assure uniform cavity size, and then were finished using fissure bur (#H21, 204, size 010, Komet, USA).

Restorative procedures:

The prepared cavities were rinsed with water to remove debris and dog's teeth were isolated by cotton rolls. MMPs inhibitor was applied by plastic syringe into cavity for 1minute without use the acid etch before and spread by using micro brush of bonding system. Also, the excess MMPs inhibitor was dried by absorbent pellet of sponge, followed by gentle free air for 5 seconds. the self-etch primer (CLEARFIL SE, KURARY CO., LTD, Japan) was placed inside the cavity for 20 seconds, then dried for 10 seconds. The self-etch bond was applied to the cavity walls by a micro brush then light cured for 10second. The composite resin (Filtek Z250, 3M ESPE, U.S.A) was placed inside the cavity by teflon coated condenser (Dentsply instrument, UK) and light cured 20 seconds for each increment.

Micro-tensile bond strength measurement:

Each tooth was mounted on the cutting machine (Isomet 4000 linear precision saw, BUEHLER, Germany), and sectioned into a series of 1mm thick slabs under water cooling. Micro-tensile bond strength was measured for each sample by using (Istron, model 3345, England) universal testing machine and data were calculated and recorded using computer software (BlueHill universal).

Statistical analysis:

Numerical data was represented as mean and standard deviation (SD) values. Normality and variance homogeneity assumptions were validated

by viewing data distribution and by using Shapiro-Wilk's and Levene's tests respectively. Data were analyzed using two-way ANOVA. Comparisons of simple main effects were made utilizing the error term of the two-way model with p-values adjustment using Bonferroni correction. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.3.2 for Windows (R Core Team (2024). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria)

RESULTS

Results of two-way ANOVA presented in Table (1), showed that there was a significant interaction effect between inhibitor type and testing period on bond strength($p<0.001$). Summary statistics and comparisons of simple effects are presented in Table (2) and in Figure (2). Results showed that at both intervals, there was a significant difference between inhibitor types ($p<0.05$). For samples measured after 6 months, post hoc pairwise comparisons showed the control group and CHX to have significantly higher values than EDTA ($p<0.001$). However, for samples measured after 12 months, post hoc comparisons showed CHX to have a significantly lower value than other types ($p=0.004$). Regarding, the control group and CHX, bond strength values significantly reduced after 12 months ($p<0.001$). While for EDTA, there was no significant difference between values measured at both intervals ($p=0.728$).

TABLE (1) Two-way ANOVA test results for micro-tensile bond strength (MPa).

Parameter	Sum of squares (II)	df	Mean square	f-value	p-value
MMP inhibitor	638.79	2	319.39	**4.04**	**0.031***
Time	3882.68	1	3882.68	**49.10**	**<0.001***
Inhibitor* time	2591.16	2	1295.58	**16.38**	**<0.001***

**Significant ($p<0.05$).*

TABLE (2) Summary statistics and simple main effects comparisons.

Inhibitor / Time	Micro-tensile bond strength (MPa) (Mean±SD)			f-value	p-value
	Control	***CHX***	***EDTA***		
6 months	60.06±12.13^{A}	65.71±14.53^{A}	34.71±5.68^{B}	**17.23**	**<0.001***
12 months	32.66±5.47^{A}	22.87±2.97^{B}	36.69±6.72^{A}	**9.03**	**0.004***
f-value	**23.73**	**58.01**	**0.12**		
p-value	**<0.001***	**<0.001***	**0.728**		

*Values with different superscripts within the same horizontal row are significantly different *Significant ($p<0.05$).*

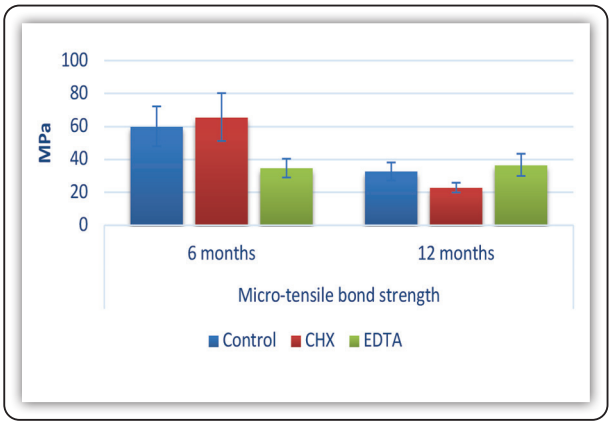

FIG (2) ANOVA test used for sample size calculation.

DISCUSSION

The integrity of adhesive systems and tooth structure dictates the lifespan and durability of resin composite restorations [18]. The degradation of resin-dentin bonds that takes place within the hybrid layer makes successful long-term dentin bonding difficult to achieve [2]. Compared to enamel, dentin has a more complex composition because of its heterogeneous morphology and organic and inorganic content [4,21]. Dentin's acid conditioning leads to micro retentions but also exposes collagen fibrils. The collagen fibrils encapsulation was not fully completed due to insufficient resin infiltration at the hybrid layer's base [6]. This collagen has not been infiltrated can be degrade by host-derived collagenolytic enzymes like matrix metalloproteinases (MMPs), and cysteine cathepsins (CCs) which can cause the resin-dentin bond to break [3].

Matrix metalloproteinases (MMPs) enzymes are a family of endo-peptidases that depend on zinc and calcium and regulate the metabolism of collagen-based tissues in both physiological and pathological conditions[22]. During tooth development, odontoblasts produce these enzymes, which are then trapped in the mineralized dentin matrix [10]. MMPs are physiologically secreted as pro-enzymes (Pro-MMPs), which are inactive. Pro-MMPs activated by various factors, such as pH fluctuations resulting from cariogenic challenges or pH changes resulting from acid etching as acidic dental adhesive monomer[4]. These enzymes' activation causes the collagen fibrils degradation and weaken the bond strength[2]. In order to reduce this activity it is recommend to use specific MMPs inhibitors such as, sodium fluoride, green tea, chlorhexidine digluconate, benzalkonium chloride, nano zinc oxide, mixture of tetracycline isomer, and ethylene diaminetetra acetic acid [8,23].

This study evaluated the effects of two MMP inhibitors (EDTA and CHX) before applying a self-etch adhesive system (CLEARFIL SE). The MMPs inhibitor application time limit of 60 seconds seems reasonable in clinical settings. This was in accordance with the application sequence used by Zheng et al, 2015 [24] in their study

The results of this study at six months testing period revealed that, there was a significant difference between values of different groups. The CHX group and control group significantly higher than EDTA group. This might be due to CHX has a strong affinity to the dental structure, binding to negatively charged carboxyl groups in the collagen matrix and positively charged phosphate groups in dentin crystallites [25]. Thus, in both mineralized and demineralized dentin, it can remain bonded. This was responsible of stable bond following CHX treatment. Although CHX is believed to chelate the zinc and calcium ions which were required to activate released MMPs enzymes. In the absence of these ions, MMPs enzymes kept in their inactive form and the collagen fibrils were not degraded [10].

This result was agreement with Tjäderhane et al, 2013 [3] and Rabeia et al, 2015 [26].On the other hand, these results were contradicted by Ebrahimi et al, 2022[27] who found that CHX molecule are water soluble and may be gradually leached out from the adhesive interface. Furthermore, the application of CHX might be changing resin's capability to seal dentin. So, it might affect negatively on the infiltration of the resin [18].

Results of the study showed that EDTA has lower significant effect on micro tensile bond strength than CHX and control groups. This may be due to the EDTA is water soluble, hence it might not be able to sustain MMPs inhibition for long duration such as the 6 months' period This result was in accordance with previous studies [28, 29]. However, this result was in contradiction to Tekçe et al, 2016 [19], who found the use of EDTA can increase the bond durability of mild adhesives. This result may be due to difference in the type of adhesive system and concentration of EDTA used in this study.

The results of twelve months of testing period revealed that, the value of EDTA group was significantly higher than value of CHX group. Also, there was no significant difference between control and EDTA groups. This result might be due to capability of EDTA on prevention of H^+ induced conformation changes, preserving the spongy character of the etched collagen matrix and consequently improving resin infiltration. These finding were in agreement with Kasraei et al, 2013[30] and disagreement with Matos et al, 2017 [28].

Effect of time on MMPs inhibitors showed there was a significant decrease in micro-tensile bond strength of all groups measured at the two testing periods. The highest value was measured at 6 months, while the lowest value was measured at 12 months. This may be due to numerous factors that affect the bond strength of adhesive agent and induce mechanical stresses such as, temperature and pH fluctuating of oral cavity, chewing forces, resin shrinkage, water sorption, and enzymatic action of MMPs. This was agreement with Dionysopulos et al, 2016 [31].

In this study, the loss of bond strength may be due to the plasticization of the adhesive might occur with time due to water absorption which lead to hydrolytical degradation of unreacted adhesive monomers [32]. This leads to decrease of bonding strength over time. Polymers undergo decreasing in the physical properties as a result of water sorption after polymerization and the extraction of these unreacted and water soluble monomers decreasing its concentration over time. This result was in agreement with Kwon et al, 2015 [33] and Betancourt et al, 2019 [2].

Another explanation to decrease of bond strength, after superficial demineralization by using an adhesive primer. Both Extrafibrillar and intrafibrillar crystallites were removed, exposing matrix-bond MMPs and enabling their gradual attack and degradation of the exposed collagen fibrils at the base of the hybrid layer [34]. These unprotected fibrils are created due to gradual decrease of monomer impregnation with the depth of fibrils that mean the base of hybrid layer is less infiltrated with resin leading to zones of un infiltrated collagen network in the hybrid layer [5].

The dentin bonding is not as previously assumed, and that there are two major mechanism involved in degradation of dentin-resin interface over time. The first mechanism is slow hydrolysis of the resin component caused by water sorption and the effect of salivary esterase. The second mechanism is degradation of the water rich and resin spare denuded collagen fibrils within hybrid layer, caused by activation of MMPs during bonding procedures[35].

CONCLUSIONS

Within the limitations of this in vivo study, we can be concluded that the application of EDTA as MMP inhibitors improve micro-tensile bond strength after 12 months of aging, while the bond strength decreased by aging for CHX and control groups.

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