



Effect of Trypsin/EDTA Conditioning on Marginal Microleakage of Class V Composite Restorations Bonded With a One-Step Self-etch Adhesive

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Abstract

Background: Sodium hypochlorite and EDTA solutions are used in the preparation of dentin surfaces, which may increase the bond strength of the composite restorations and decrease their microleakage scores. This in-vitro experimental study aimed to assess the effect of dentin surface conditioning with EDTA/trypsin solution prior to bonding procedure with Clearfil S3 Bond on the microleakage of class V composite restorations.

Methods: Class V cavities were prepared on the buccal and lingual surfaces of 18 human premolars (n=18) and randomly assigned into two groups. In the control group, Clearfil S3 Bond was applied on the dentin surfaces. While in the experimental group, before applying Clearfil S3 Bond, the surfaces were conditioned with EDTA/trypsin solution. The cavities on the teeth were then incrementally filled with Filtek Z250 light-cure composite. Afterward, all the samples were thermocycled for 500 cycles. Next, the specimens were immersed in methylene blue solution for 72 hours. The restorations were sectioned buccolingually through the center of the restorations with a diamond disk. Then, microleakage at the tooth-restoration interface was evaluated in the enamel and dentin margins blindly using dye penetration under a stereomicroscope at $\times 40$ magnification. Finally, data were statistically analyzed by Mann-Whitney U test.

Results: The results showed no significant differences between two groups regarding the microleakage scores of the enamel and dentin edges. In addition, in all sections and edges of the experimental group, decreased microleakage scores were attained compared to the control specimens ($P < 0.03$).

Conclusions: Regarding the limitations of the present study, the preparation of dentin surfaces with EDTA/trypsin solution before applying Clearfil S3 Bond resulted in the decreased microleakage scores compared to unprepared surfaces.

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Keywords: EDTA,
Microleakage, One-stage
bonding system, Trypsin
solution

Received July 23, 2018

Accepted Sep. 3, 2018

ePublished Sep. 12, 2018



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Citation: Kasraei S, Kordestani M, Yarmohammadi E, Pashaei H. Effect of trypsin/EDTA conditioning on marginal microleakage of class V composite restorations bonded with a one-step self-etch adhesive. Avicenna J Dent Res. 2018;10(3):101-106. doi: 10.34172/ajdr.2018.21.

Background

Adhesion to tooth structure depends on several factors, including the type of adhesive system, tooth structure, cyclic loading, and surface contaminations (1). Bonding failure of resin-dentin composite decreases retention, allows microleakage, and results in the development of recurrent caries (2). Contemporary one-step self-etch adhesives contain three components of conditioner, primer, and adhesive agent all in one solution (3). Self-etch systems relatively simply, bond to dentin and are less technique sensitive. Absence of sensitivity or minimal postoperative sensitivity is another important clinical advantage of self-etch systems (4). On the other hand, smear layer is a crucial factor, affecting the adhesion of composite to tooth structure (3). It is formed on dentin

Highlights

- ▶ Adhesion to tooth structure depends on several factors, including the type of adhesive system, tooth structure, cyclic loading, and surface contaminations.
- ▶ It has been proposed that use of a chelating agent such as ethylene diamine tetra acetic (EDTA) acid as a conditioner can enhance the bond strength of mild self-etch systems to dentin.
- ▶ Microleakage at enamel margins of the restorations is significantly lower than that at dentin margins.
- ▶ It seems that microleakage of composite restorations can be decreased by the use of trypsin.
- ▶ Application of EDTA/trypsin prior to the use of one-step self-etch S3 Bond can significantly decrease the marginal microleakage of class V composite restorations.

surface following the clinical use of diamond or tungsten carbide burs (5). Its presence can decrease the diffusion of

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self-etching primer into the underlying dentin (6). Several materials have been introduced for smear layer removal. Phosphoric acid is one of the most common materials used for this purpose (7). Some previous studies have shown that the use of phosphoric acid prior to the application of self-etch adhesives on dentin surface decreases the bond strength and increases microleakage at dentin margins of composite restorations (4). Thus, it has been proposed that use of a chelating agent such as ethylene diamine tetra acetic (EDTA) acid as a conditioner can enhance the bond strength of mild self-etch systems to dentin (4,8,9). Moreover, it has been indicated that EDTA inhibits matrix metalloproteinases (MMPs) and improves the durability of resin-dentin bond (10).

The superficial smear layer contains both mineral and organic components, which can affect the penetration of adhesives through underlying dentin (11,12). Several methods have been suggested for decreasing the protein materials present in dentin surface. Previous studies have confirmed that application of a proteinase to eliminate these organic materials, can enhance adhesion to dentin as well as resulting in better diffusion of bonding agents into the bonding interface (6-8).

Trypsin is an amino acid protease, which can be used as a proteolytic agent to decrease or eliminate the organic components (13,14). It seems that microleakage of composite restorations can be decreased by the use of trypsin (15). Taking the materials aforesaid in mind, this study aimed to assess the effect of EDTA/trypsin conditioning prior to the application of Clearfil S3 Bond (the one-step adhesive) on the marginal microleakage of class V composite restorations. The null hypothesis was that the use of EDTA/trypsin prior to the application of a one-step self-etch adhesive system would have no effect on marginal leakage in class V composite restorations.

Methods

The current in vitro experimental study was conducted on human premolar teeth extracted within the past three months for orthodontic reasons. The teeth were stored in 10% buffered formalin after extraction. One week prior to the experiment, the teeth were evaluated under a stereomicroscope (Olympus, Tokyo, Japan) at $\times 16$ magnification, from which 18 teeth free from caries, fracture, wear, restoration, cracks, and congenital anomalies were chosen. After cleaning the teeth from debris and soft tissue residues, they were stored in distilled water at room temperature.

The teeth were then randomly divided into two groups. Classic class V cavities were prepared on the buccal and lingual surfaces of the teeth at the cemento-enamel junction (CEJ) using high speed handpiece and fissure diamond burs (835008, Tizkavan, Tehran, Iran) with a diameter of 1 mm under air and water spray. Finally there were 18 cavities in each group. The prepared cavities had a dimension of 2 mm depth, 2 mm width, and 3mm length,

in such a way that 1mm of the width of the cavity was above the CEJ and in the enamel and 1mm of the cavity width was below the CEJ and in the cementum or dentin. Enamel margins were beveled for 0.5-0.75 mm width at 45-degree angle. The burs were replaced after preparing 9 cavities. Thus, each tooth had two cavities: one in the buccal and one in the lingual surface. A total of 36 classic class V cavities, therefore, were prepared and restored as follows:

Control group: One layer of Clearfil S3 Bond (Kuraray Medical Inc., Tokyo, Japan) was applied on dentin surface for 20 seconds according to the manufacturer's instructions. Excess bonding agent was eliminated by air spray for five seconds. Light curing was carried out for 10 seconds using a light curing unit (Demi™ Plus; Demetron Kerr, Middleton, WI, USA) at light intensity of 1100 mW/cm².

Experimental group: The teeth were prepared as in the control group except that prior to the application of bonding agent, dentin surface was conditioned with EDTA/trypsin solution for 30 seconds. Trypsin solution used in this study was prepared by adding 0.5 wt% trypsin (Heissner, MERK, Germany) and 0.2 wt% disodium EDTA-2H₂O (Heissner, MERK, Germany) to Hanks' balanced salt solution without Mg and Ca. For cavity surface conditioning, EDTA/trypsin solution was rubbed on prepared tooth surfaces for 15 seconds using a microbrush and remained as such for 15 seconds with no agitation. Then, the samples were rinsed with water spray for 15 seconds and air dried. Clearfil S3 Bond was then applied according to the manufacturer's instructions. The cavities were filled with Filtek Z250 light-cure composite (3M ESPE, St. Paul, MN, USA) in two oblique increments. Each increment was light cured for 20 second. The restorations were finished with fine grit finishing burs (Isomet, BuehlerLtd, LakeBluff, IL, USA), then polished using Soflex discs (#1982, 3M Dental Product Division, St. Paul, MN, USA). Afterward, the specimens were stored in distilled water at room temperature for 24 hours, and were then thermocycled for 500 cycles between $5\pm 1^\circ\text{C}$ and $55\pm 1^\circ\text{C}$. Each thermal cycle lasted for 80 seconds and included 30 seconds at 5°C , 30 seconds at 55°C , and 20 seconds at room temperature (24°C). The root apices were sealed with sticky wax. Next, all surfaces of each tooth were covered with two layers of nail varnish except for 1 mm margin around the restoration. After drying the varnish, the specimens were immersed in 10% methylene blue for 72 hours. The teeth were then rinsed with water and blot-dried.

In the next step, the teeth were sectioned buccolingually through the center of the restorations, using two-sided diamond discs (Isomet, BuehlerLtd, LakeBluff, IL, USA) under water. Two blind observers examined the enamel and dentin margins of the two sections of each tooth under a stereomicroscope (SZ40, Olympus, Tokyo, Japan) at $\times 40$ magnification. The highest score of microleakage

was recorded for each sample. Microleakage was scored as follows: 0- No dye penetration; 1- Dye penetration up to half of the cavity depth; 2- Dye penetration more than half of the cavity depth, but not extending the axial wall; 3- Dye penetration arriving to the cavity floor/axial wall and beyond.

Statistical Analysis

Microsoft Excel spreadsheet (Microsoft Office Excel 2007) was used for statistical evaluation, as well as using the SPSS software version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Non-parametric Mann-Whitney U test was also employed to analyze the differences in microleakage scores between the two groups at the enamel and dentin margins on the buccal and lingual surfaces. Furthermore, microleakage scores at the enamel and dentin margins within each group were compared using Wilcoxon signed rank test. Finally, type one error was considered as $\alpha=0.05$ and $P<0.05$ was considered statistically significant.

Results

According to the results, comparison of total marginal microleakage scores in class V composite restorations in the two groups revealed significantly lower microleakage score in the experimental group conditioned with EDTA/trypsin compared to the control group (Table 1, $P=0.03$). The Mann-Whitney U test revealed no significant difference in microleakage scores between the control and experimental groups at the occlusal (enamel) margins ($P=0.13$). Although the microleakage score at the gingival (dentin) margins in the EDTA/trypsin group was slightly lower, the difference in this regard between the two groups was not significant ($P=0.18$). The Wilcoxon

signed rank test showed that the microleakage score at occlusal (enamel) margins was significantly lower than that at the gingival (dentin) margins (Table 2, $P=0.03$).

Discussion

Marginal microleakage is cited as one of the most common reasons for the failure of composite restorations. Electron microscopy is the most accurate method for the assessment of microleakage (16). However, dye penetration test has been an easy and useful method for the assessment of marginal microleakage in many studies (17).

This study aimed to assess the effect of surface conditioning with EDTA/trypsin protease on the marginal microleakage of class V composite restorations. The results showed that application of EDTA/trypsin prior to the use of one-step self-etch S3 Bond significantly decreased the marginal microleakage of class V composite restorations ($P=0.03$). This finding corroborated that of some previous studies (1,8,15).

In self-etch systems, acidic monomers dissolve the smear layer, but the dissolved smear layer and surface demineralization products are not rinsed in a separate step. Thus, the smear plugs are not completely eliminated from the orifice of dentinal tubules. The smear layer dissolved by the acidic monomers remains as part of the hybrid layer. This process can prevent optimal and efficient function of one-step adhesive systems (18,19). It has been argued that separate conditioning of the cavity enhances resin penetration into the interfibrillar spaces (4,6).

EDTA is a conditioner, which can eliminate the smear layer without causing any major alteration in the structure of the collagen matrix of underlying dentin. In addition,

Table 1. Frequency Distribution of Microleakage Scores at the Enamel and Dentin Margins of the Experimental and Control Groups

Margins	Groups	Microleakage				P Value*
		Score 0	Score 1	Score 2	Score 3	
Enamel margin	Control	6 (33.3%)	11 (61.1%)	1 (5.6%)	0 (0%)	0.13
	Experimental	11 (61.1%)	7 (38.9%)	0 (0%)	0 (0%)	
Dentin margin	Control	4 (22.2%)	10 (55.6%)	3 (16.7%)	1 (5.6%)	0.18
	Experimental	8 (44.4%)	8 (44.4%)	2 (11.1%)	0 (0%)	
All margins of restorations	Control	10 (27.8%)	21 (58.3%)	4 (11.1%)	1 (2.8%)	0.03
	Experimental	19 (52.8%)	15 (41.7%)	2 (5.6%)	0 (0%)	

* Mann-Whitney U test.

Table 2. Frequency Distribution of Microleakage Scores at the Enamel and Dentin Margins of the Study Groups

Margins	Microleakage				P Value*
	Score 0	Score 1	Score 2	Score 3	
Occlusal margin	17 (47.2%)	18 (50.0%)	1 (2.8%)	0 (0%)	0.03
Gingival margin	12 (33.3%)	18 (50.0%)	5 (13.9%)	1 (2.8%)	

*Wilcoxon signed rank test.

EDTA molecule contains 4 carboxylic acid groups capable of selective elimination of hydroxyapatite crystals. Moreover, it has been demonstrated that EDTA inhibits MMPs and can enhance the stability and durability of resin-dentin bond (10).

Li et al in 2015 revealed that treatment of dentin surface with EDTA prior to bonding improved the bond strength and prevented marginal discoloration in cervical wedge-shaped lesions (8). In addition, Kasraie et al in 2013 reported that application of EDTA at 0.5M concentration prior to the use of Clearfil S3 Bond significantly increased the bond strength of restorative material (2). Furthermore, Stape et al in 2012 evaluated the effect of 24% EDTA on bond strength of resin cements to tooth structure and concluded that the positive effects of EDTA on bond strength depended on the type of resin cement (20). EDTA eliminates the smear layer and enhances the penetration of acidic resin monomers into the intertubular dentin beneath the smear layer (21,22).

However, some previous studies reported that chelating agents such as EDTA were not effective in prevention of marginal microleakage in composite restorations. For example, Singh et al in 2015 reported that treatment with EDTA prior to the use of self-etch adhesive systems had no significant effect on the durability of resin-dentin bond (4). The concentration of EDTA, method of application, and duration of dentin surface conditioning in the study of Singh et al were different from those in the present study. It has been shown that the effects of EDTA solution depend on several factors including the pH value, concentration of material, and duration of application (23). In the current study, 0.2 M EDTA along with 0.5% trypsin was used. Trypsin as a non-specific endopeptidase cleaves the protein chains at the lysine-arginine bond into small peptides (13,14). Surface conditioning with trypsin as a proteolytic factor can be performed to decrease or eliminate the residual organic components (24).

According to the study of Lu et al in 2014, use of trypsin increases the thickness of hybrid layer when two-step etch-and-rinse adhesives are used. They added that following the dentin surface conditioned with trypsin may lead to an increase in the penetration of adhesive monomers into the demineralized dentin matrix and dentinal tubules (15).

In order to eliminate organic materials from the cavity surface and improve the quality of bond to tooth structure, some studies have suggested the use of sodium hypochlorite and reported controversial results (25-27). Residual sodium hypochlorite may interfere with the polymerization of resin after rinsing the conditioned surface and facilitate the creation of a fragile zone in the hybrid layer (28).

Chauhan et al in 2015 evaluated the effect of deproteinization of dentin surface with bromelain enzyme, which belongs to the group of proteolytic

enzymes (29), and 5% sodium hypochlorite on shear bond strength of composite restorations (1). They found that the bond strength in the group subjected to treatment with bromelain enzyme was significantly higher than that in the control and sodium hypochlorite groups (1).

However, in some other studies, dentin surface conditioning with trypsin did not enhance the bond strength of restorative material to tooth structure. Bedran-Russo et al in 2008 displayed that surface conditioning with trypsin decreased resin-dentin bond strength. However, it should be noted that in their study, two-step fifth generation adhesive system was used and the trypsin solution was provided by the manufacturer (Worthington Biochemical Corp.) (30).

The results of the current study revealed that the microleakage at enamel margins of the restorations were significantly lower than that at dentin margins ($P<0.05$). This finding was in agreement with the results of most previous studies (31-33).

Enamel etching with acids such as phosphoric acid, which is conventionally used in enamel bonding, results in the creation of three different microscopic patterns. In general, the bond of adhesive systems to enamel is more reliable than that to dentin, which is explained by more complex histology and less mineral content of dentin compared to enamel. Furthermore, the crystallization pattern of enamel crystals is more organized compared to dentin (34). Enamel etching by one-step self-etch systems is not as efficient as separate enamel etching using the phosphoric acid (in a separate step), and despite fewer procedural steps and lower technical sensitivity, some concerns still exist with regard to the application of self-etch compared to etch-and-rinse systems (35). The thin prismless enamel layer present in the cervical region of tooth crown is responsible for lower bond strength to resin. It can be a reason for occlusal marginal microleakage of class V restorations in this area. However, it should be noted that enamel margin beveling prevents enamel crazing compared to a butt joint (90°) finish line, and might play a major role in decreasing the microleakage of occlusal margins of restoration.

Conclusions

Considering the limitations of the present study, cavity surface conditioning with 0.5% trypsin/EDTA prior to the application of Clearfil S3 Bond can significantly decrease the marginal microleakage of class V composite restorations.

Authors' Contribution

All authors have contributed to the concept and design of the study. SHK and EY supervised the conduct of the experiment. HP contributed to the data collection. The statistical analyses and interpretation of data were carried out by MK. MK also drafted the manuscript. All the authors critically revised the manuscript for intellectual content. All the authors have read and approved the final manuscript.

Ethical Statement

This study was approved by the Ethics Committee of the Vice Chancellor for Research, Hamedan University of Medical Sciences, Hamadan, Iran.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Acknowledgments

The authors thank the Dental Research Center and the Vice Chancellor for Research at Hamedan University of Medical Sciences for supporting this study.

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