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ORIGINAL ARTICLE

Bacterial microleakage of aged adhesive restorations

Nevin Cobanoglu, Emine Kara[1], Nimet Unlu[2], Fusun Ozer[3]

Department of Restorative Dentistry, Faculty of Dentistry, University of Selcuk, Konya, [1]Department of Restorative Dentistry, Faculty of Dentistry, University of Yüzüncü Yıl, Van, Turkey, [2]Department of Restorative Dentistry, Faculty of Dentistry, University of Necmettin Erbakan, Konya, Turkey, [3]Department of Preventive and Restorative Sciences, University of Pennsylvania, Philadelphia, USA

Address for correspondence:
Dr. Nevin Cobanoglu,
Department of Operative Dentistry,
Selcuk University Faculty of Dentistry,
Campus, Konya 42075, Turkiye.
E-mail: nevin_ceylan@hotmail.com

ABSTRACT

Objective: The aim of this study was to investigate the marginal bacterial leakage of two self-etch adhesive systems after long-term water storage. **Materials and Methods:** Class V cavities were prepared on the buccal and lingual surfaces of extracted premolar teeth. After the sterilization of the teeth, four cavities were not restored for control purposes, whereas the other teeth were divided into two groups (n = 16 cavities each): Clearfil Protect Bond (CPB), Clearfil SE Bond (CSE). After the application of the bonding agent, cavities were restored with a composite resin. Then, the teeth were thermo cycled, stored in saline solution for 6 months and put into a broth culture of *Streptococcus mutans*. The teeth were fixed, sectioned and stained using the Gram-Colour modified method. The stained sections were then evaluated under a light microscope. The bacterial leakage was scored as: 0 - absence of stained bacteria, 1 - bacterial staining along the cavity walls, 2 - bacterial staining within the cut dentinal tubules. The data were analysed using the Kruskal–Wallis and Mann–Whitney U-test (P = 0.05). **Results:** The bacterial staining was detected within the cut dentinal tubules in all control cavities, in three cavities in the CSE group and one cavity in the CPB group. There were no observed statistically significant differences between the bacterial penetrations of the two bonding systems (P > 0.05). **Conclusion:** Both bonding systems provided acceptable prevention of marginal bacterial leakage after long-term water storage.

Key words
Antibacterial adhesive, bacterial microleakage, self-etching adhesive

INTRODUCTION

A hermetic seal against microleakage is very important, since the bacteria in the tooth/restoration interface are the main cause of secondary caries and pulp damage. Despite the fact that the use of dentin bonding systems, with their micromechanical adhesion to tooth structure, has greatly reduced microleakage, most of these bonding systems have not been proven to be completely effective in eliminating microleakage at the tooth restoration interface.[1-3] In addition, *in vitro* studies on the degradation of dentin/resin bonds support that progressive decreases in bond strengths occur after aging.[4,5] Once the bond between the tooth/restoration is degraded, and a gap is formed, bacteria and their toxic products readily invade. Therefore, it may be very important to use adhesive systems having antibacterial effects that are able to reduce bacterial invasion and growth during the time periods following the restoration of cavities. However, the antibacterial activity of adhesive systems seems to be dependent on their acidity and chemical composition, which can be suppressed after light-activation.[6-8]

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Clearfil Protect Bond (CPB) is a self-etching adhesive system containing 12-methacryloyloxydodecyl-pyridinium bromide (MDPB), which has antibacterial activities. It has been demonstrated that the adhesive system incorporating MDPB can show antibacterial effects before and after curing.[9-11]

The aim of this study was to investigate the marginal bacterial leakage of two self-etch adhesive systems, containing MDPB (CPB) and not containing MDPB Clearfil SE Bond (CSE), after long-term water storage. The null hypothesis is that aged adhesive restorations cannot provide inhibition of the marginal bacterial leakage.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Faculty of Dentistry, University of Selcuk, Konya,

Turkey, and consent was obtained from the patients to retain and use their teeth. The teeth were cleaned, stored at 4°C in 0.1% tymol, and were used within 1-month following extraction.

Eighteen noncarious human premolar teeth were used. After cleaning with a rubber cup and slurry of pumice, two standardized Class V cavities were prepared on the buccal and lingual (palatal) surfaces of the teeth using a diamond bur (M and A Dıatek, 110 314 110 534 012M) at ultra-high speeds with a copious water spray. The dimensions of each preparation were approximately 3 mm wide × 2.5 mm deep × 2 mm long occlusal-cervically. A new bur was employed on every five cavities to avoid excessive heating. One-half of the cavity margin was located in the enamel, and the other half was located in the cement. The prepared teeth were then sterilized using a steam autoclave at 121°C for 15 min, and randomly assigned to the three groups where the cavities were treated as follows:

- 2 teeth (4 cavities) without restoration for control
- 8 teeth (16 cavities) with CSE (Kuraray, Japan)
- 8 teeth (16 cavities) with MDPB-containing CPB (Kuraray, Japan).

Bonding procedures were performed according to the manufacturer's instructions [Table 1]. Then, the cavities (with bonding) were restored using a hybrid restorative resin composite (Clearfil APX, Kuraray, Japan), using an aseptic technique, under a laminar air flow hood. After finishing and polishing, the teeth were submitted to thermocycling (Nova, Konya, Turkey) 1000 times at 5–55°C, with a 15 s dwell time in sterile physiological saline (SPS), and stored in SPS at room temperature for 6 months. At the end of the storage period, the entire tooth surfaces, except for the restoration and 1 mm around, were covered with two layers of nail polish. The root tips were also sealed with bonding agents and composite materials. The teeth were stored in a broth culture of 1.56×10^8 CFU/ml of *Streptococcus mutans* at 37°C for 10 days, allowing bacterial leakage into the cavity margins. The broth culture was changed twice per week.

After incubation, the nail polish was removed, and the teeth were fixed in a 10% neutrally-buffered formal saline solution for 48 h. The teeth were decalcified in 5% nitric acid and then washed thoroughly in running water for 18 h, dehydrated and embedded in paraffin. Serial sections of 7 μm thick were prepared from each tooth using a microtome, and bacterial staining was done using the Gram-Colour modified method (Merc). Finally, 20 serial sections from each tooth were evaluated under a light microscope twice, on a blinded basis, by two independent observers. Bacterial leakage was recorded according to the following criteria: 0 - absence of stained bacteria, 1 - positive bacterial staining in cavity walls and floor, and 2 - positive bacterial staining within the cut dentin tubules. Ordinal data were statistically analysed using the Kruskal–Wallis and the Mann–Whitney U-test.

RESULTS

The results of the bacterial micro leakage of the groups are shown in Table 2. Some bacterial staining along the cavity wall and floor were observed in three cavities from the CPB group and five cavities from the CSE group [Figure 1]. Additionally, bacterial staining within the cut dentinal tubules was seen in all control cavities, one cavity of the CPB group and three cavities of the CSE

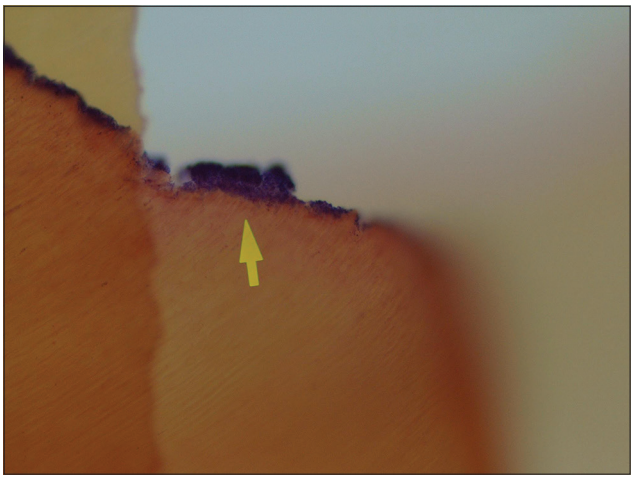

Figure 1: The cavity without restoration in control group, bacteria were obsedved in cavity floor and within the dentin tubules (Gram-Colour modified method ×400)

Table 1: Application procedures, composition, pH and batch numbers of the used adhesive systems

Adhesive-manufacturer	Application procedure	Composition	pH	Batch number
CSE (Kuraray Noritake Dental, Japan)	Apply primer for 20s. Air gently, apply bonding resin, light curing for 10s	Primer: HEMA, MDP, hydrophilic dimethacrylate, N, N-diethandiol-p-toluidine, CQ, water	Primer: 1,9	00195A
		Adhesive: HEMA, MDP, hydrophilic dimethacrylate, N, N-diethandiol-p-toluidine, CQ, silanized colloidal silica, BisGMA	Adhesive: 2,8	00193A
CPB (Kuraray Noritake Dental, Japan)	Apply primer for 20s. Air gently, apply bonding resin, light curing for 10s	Primer: HEMA, MDP, hydrophilic dimethacrylate, MDPB, water	Primer: 1,9	0012A
		Adhesive: HEMA, MDP, hydrophilic dimethacrylate, N, N-diethandiol-p-toluidine, CQ, silanized colloidal silica	Adhesive: 2,8	0020A

BisGMA - Bisphenol A glycidyl dimethacrylate, CQ - D, 1-Camphorquinone, HEMA - 2-Hydroxyethyl methacrylate, MDP - 10-Methacryloxydecyl dihydrogen phosphate, MDPB - 12-Methacryloyloxydodecyl-pyridinium bromide, CSE - Clearfil SE Bond, CPB - Clearfil Protect Bond

group [Figure 2]. The differences between the marginal bacterial leakages of the two bonding systems were not statistically significant ($P > 0.05$).

DISCUSSION

The water storage and thermocycling procedures are widely accepted *in vitro* techniques used to predict the behaviour of resin restorations.[12,13] These aging procedures were used in the present study.

Previous *in vitro* studies have indicated that resin-dentin bonds degrade after long-term water storage.[14,15] The bond deterioration by water storage is the result of the degradation of the interface components, such as the denaturation of collagen and/or elution of degraded or insufficiently cured resin.[16,17]

Although the spaces in tooth-restoration interfaces are sufficiently large to allow microbial spread, some factors, such as the availability of nutrients and the antibacterial properties of the material, influence the leakage involving bacteria.[18] Therefore, leakage studies involving bacteria in assessing microleakage are more appropriate.

Previous studies have demonstrated that the microleakage of oral bacteria around restorations allows for the bacterial invasion of exposed dentinal tubules at the base of the cavity.[19,20] It has been reported that the composition of the microflora invading exposed noncarious dentin probably resembles the composition of the biofilm infiltrating the tooth-restoration interface and *Streptococcus* is one of the main microorganisms of this biofilm, which resembles mature plaque.[21-23] Therefore, in the present study, the teeth were stored in a broth culture of 1.56×10^8 CFU/ml of *S. mutans* at 37°C for 10 days, allowing bacterial leakage into the cavity margins, while the broth culture was changed twice per week.

Table 2: The results of bacterial leakage

Groups	*n*	0	1	2
CPB	16	10	3	1
CSE	16	9	5	5
Control	4	0	0	4

CPB - Clearfil Protect Bond, CSE - Clearfil SE Bond

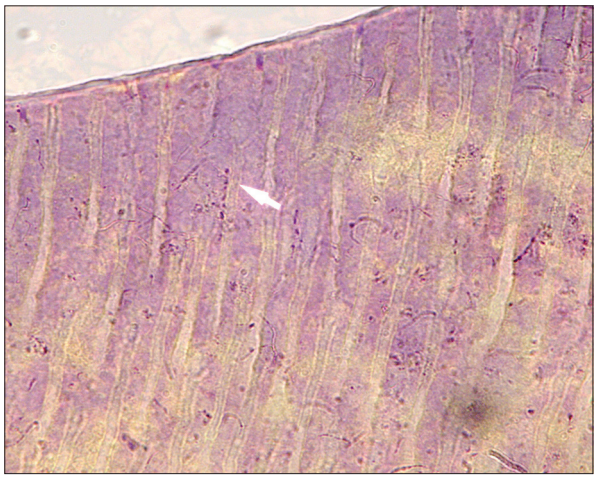

Figure 2: The cavity restored with Clearfil SE Bond, bacteria were obsedved in dentin tubules (Gram-Colour modified method ×1000)

In the present study, the effects of CSE and CPB on bacterial leakage were compared using bacterial staining techniques on histological sections after the samples were stored in water for 6 months. The majority of the teeth in both adhesive system groups were shown to have acceptable, marginal sealing against the culture of *S. mutans*. The CSE group had a greater number of teeth with bacterial staining within the cut dentinal tubules than the CPB group, although it was not statistically significant.

In some studies, the CSE and CPB produced similar bonding interfaces.[24,25] Kubo *et al.* and Siso *et al.* previously reported that CPB and CSE have the same, as well as good sealing ability.[26,27] However, Dönmez *et al.* observed that both the *in vivo* and *in vitro* specimens restored with CSE and CPB, after 12 months of aging, exhibited water trees that were not initially observed in the adhesive layers at 24 h.[4] They reported that these water channels were present in both adhesives, suggesting that a common degradation mechanism existed in these adhesives.

Despite this similar degradation in the resin-dentin interface, Dönmez *et al.* reported an increase in the bond strength of the CPB after 12 months of water storage, but a decrease in the bond strength of the CSE.[4] In other studies, after long-term water storage, increases in the bond strengths of CPB[28-30] and decreases in the microleakage of CPB at the dentinal margin were reported.[31] The results related to the CPB were attributed to the fluoride ions and antibacterial monomer (MDPB) in its contents.[3,29,32] When fluoride-containing adhesives are placed directly in contact with the cavity wall, the fluoride ions penetrating into the dentin enhance mineralization and reduce the demineralization of the dentin.[33] Additionally, the antibacterial monomer (MDPB) in the CPB possesses anti-matrix metalloproteinase (MMP) activities.[32] The MMPs trapped within the mineralized dentin matrix can be activated by modern self-etch and etch-and-rinse adhesives[34,35] and can hydrolyse the organic matrix of demineralized dentin.[36-38] After applying the adhesive system to the dentin, the exposed collagen fibrils at the bottom of the hybrid layer that result from the imperfect resin impregnation of the demineralized dentin matrix may be affected by the dentin MMPs, which may result in a reduced bond strength.[39]

Some factors, such as pH, ion release (e.g. fluoride)[6,7] or the inclusion of antibacterial monomers (MDPB), may give

antibacterial properties to adhesive systems.[11,40] Since a hermetic seal cannot be achieved at the tooth-restorations interfaces, it would be beneficial if the restorative materials could exert some antibacterial activity that was as long as the function of the restoration in the oral cavity. Therefore, it should be examined whether the antibacterial properties of adhesive systems have immediate or long-term effects.

The primer component of the CSE and CPB has an acidic pH (<2.0) that causes the antibacterial effects against most cariogenic bacteria.[41] This antibacterial effect is due to the entrance of high levels of H + protons into the cell cytoplasm. As a result, the loss of activity of the glycolytic enzymes, which are used to produce adenosine triphosphate, and the structural damage of the cell membrane and macromolecules, such as the DNA and proteins, a culmination in cell death occurs.[42] However, the antibacterial effects caused by low pH are limited, because acidic monomers released from the primer may have been neutralized by the buffer capacity of tooth tissue.[43] Besides, since the polymerization of adhesive materials decreases the release of acidic monomers, the antibacterial activity of adhesive systems is reduced after light-activation.

Duque *et al.* reported that the uncured primer components of CBP and CSE showed an inhibitory effect against *S. mutans*; but when cured specimens were placed on the agar medium, only the primer component of the CBP maintained its antibacterial activity, although significantly reduced when compared with the uncured specimens.[44] Another study has shown that a dentin primer incorporating MDPB could show antibacterial activity, before and after curing, against oral bacteria such as *S. mutans*.[6,9] The antibacterial agent is immobilized in the polymer network by the polymerization of MDPB, and the bacterial growth inhibitory activity of an MDPB-containing material after curing is exerted by direct contact with its surface.[45]

It is expected that the long-term durability of the bond at the tooth-restoration interface, and antibacterial activity of adhesive systems are beneficial in inactivating bacteria that invade the tooth-restoration interface by microleakage. It can be considered that this expectation has been fulfilled by the results of the present study: In the CSE group, bacterial staining within the cut dentinal tubules of more teeth were seen than in the CPB group, although it is not statistically significant.

CONCLUSION

Within the limitations of this *in vitro* study, both adhesive systems were shown to have acceptable, marginal sealing against bacterial leakage. Therefore, our hypothesis was rejected. The limited aging procedures used in the present study did not cause the marginal bacterial leakage of the composite restorations performed with CSE and CPB. Further research is needed in order to evaluate the long-term antibacterial effects of MDPB.

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