

Bacterial penetration of restored cavities using two self-etching bonding systems

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ABSTRACT

Objective: The aim of this study was to investigate the effects of two bonding systems, with and without antibacterial monomers, on marginal bacterial and dye leakage. **Materials and Methods:** Class V cavities were prepared in extracted teeth for a bacterial leakage test, and the teeth were sterilized using a steam autoclave. Four cavities were not restored for the controls, and the other teeth were divided into two groups ($n = 16$ cavities each): Clearfil Protect Bond group (CPB) and Clearfil SE Bond group (CSE). After application of the bonding agent, the cavities were restored using a composite resin (Clearfil AP-X). The teeth were thermocycled, stored in a broth culture of 1.56×10^8 colony forming units (CFU)/ml of *Streptococcus mutans* at 37°C for 10 days, and subsequently processed for bacterial staining. Sections from the demineralized teeth were evaluated under a light microscope. In the dye leakage test, the cavities were restored as described in the bacterial penetration test. After thermocycling, the teeth were immersed in 5% basic fuchsin for 24 h, and then divided in half and observed under a stereomicroscope. The data were analyzed using the Kruskal–Wallis and Mann–Whitney U-tests ($P = 0.05$). **Results:** The bacterial stain was detected at the cavity wall of five cavities in both bonding systems. Additionally, two cavities in the CSE group, one cavity in the CPB group, and all control cavities showed bacterial staining within the cut dentinal tubules. Dye staining at the axial cavity wall was detected in only three of the teeth for both bonding systems. **Conclusion:** The bonding systems used in this study provided an acceptable marginal seal to prevent bacterial and dye leakage.

Key words: Antibacterial adhesive, bacterial microleakage, dye leakage, self-etch adhesive

INTRODUCTION

The polymerization shrinkage of composite resins may lead to the formation of gaps at the tooth–restoration interface.^[1] Additionally, the oral cavity, with its associated temperature changes, chewing loads, and chemical attacks by acids and enzymes, creates a rather severe challenge for tooth composite bonds. The degradation of bonding at the tooth–restoration interface and the formation of gaps can result in the passage of bacteria, fluids, or ions between the cavity wall and the resin composite, a process known as

microleakage.^[2] Many new bonding systems have been introduced for reducing the microleakage of resin composite restorations; however, most of these bonding systems have not been proven to be completely effective in eliminating microleakage at the tooth–restoration interface.

The pulpal reactions observed after filling cavities are mainly due to the passage of bacteria between the dentinal walls and the filling material, toward the pulp.^[3] The long-lasting antibacterial activity of the polymerized adhesives may also be effective

How to cite this article: Cobanoglu N, Ozer F, Demirci M, Erganis O, Imazato S. Bacterial penetration of restored cavities using two self-etching bonding systems. *Eur J Dent* 2014;8:166-71.

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DOI: 10.4103/1305-7456.130590

in inactivating the bacteria that invade the tooth-adhesive interface through microleakage, and the use of these adhesive systems may prevent the passage of bacteria toward the pulp.

Testing for microleakages along the dentin-restoration interface using organic and inorganic dyes has been widely used due to its speed in obtaining results and its ease of execution.^[4,5] However, this test is not appropriate for evaluating leakage involving bacteria. Although the space appears to be sufficiently large to allow microbial spread with all materials, other factors, such as the availability of nutrients and the antibacterial properties of the material, influence the leakage involving bacteria.^[6] These observations suggest the importance of leakage studies involving bacteria in assessing microleakage.

The aim of this study was to investigate the bacterial penetration of restored cavities with two bonding systems, with and without antibacterial monomers. The null hypothesis was that there is no difference between the bacterial microleakage of the bonding systems with or without the antibacterial monomer.

MATERIALS AND METHODS

The teeth used in the present study were collected with the patients' informed consent under a protocol reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Selcuk University. The teeth were stored at 4°C in 0.5% chloramine water, and were used within 1 month following extraction.

Bacterial penetration test

Eighteen non-carious human wisdom teeth were used in this study. After surface debridement with a hand scaling instrument and cleaning with a rubber cup and slurry of pumice, two standardized Class V cavities (approximately 3 mm wide × 2 mm deep × 2 mm long) were prepared on the buccal and lingual surfaces

of the teeth using a diamond fissure-shaped bur (M and A Diatek, 110 314 110 534 012M) at ultra-high speeds with a copious water spray. A new bur was employed on every fourth cavity to avoid excessive heating. One half of the cavity margin was located in the enamel and the other half in the cement. The prepared teeth were sterilized using a steam autoclave at 121°C for 15 min and randomly assigned to one of the two groups. The cavities were treated as follows:

1. 4 cavities in 2 teeth without restoration for control
2. 16 cavities in 8 teeth with Clearfil SE Bond (CSE) (Kuraray, Osaka, Japan)
3. 16 cavities in 8 teeth with 12-methacryloyloxy-dodecyl pyridinium bromide (MDPB)-containing Clearfil Protect Bond (CPB) (Kuraray, Osaka, Japan).

After the application of the bonding procedures according to the manufacturer's instructions [Table 1], the cavities (with bonding) were restored with a hybrid restorative resin composite (Clearfil APX; Kuraray, Osaka, Japan) using an aseptic technique, under a laminar air flow hood. After finishing and polishing, the specimens were placed in sterile physiological saline (SPS) at 37°C for 48 h. The teeth were subjected to thermocycling (Nova, Konya, Turkey) 1000 times at 5-55°C, with a 15 s dwell time in SPS. Then, the entire tooth surfaces, with the exception of the restoration and 1 mm surrounding it, were covered with two layers of nail polish. The root tips were also sealed with bonding agents and composite material. The teeth were stored in a broth culture of 1.56×10^8 colony forming units (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

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

Adhesive manufacturer	Application procedure	Composition	pH	Batch number
Clearfil SE Bond (Kuraray Noritake Dental, Japan)	Apply primer for 20 s. Air gently, apply the bonding resin, light curing for 10 s	Primer: HEMA, MDP, hydrophilic dimethacrylate, <i>N</i> , <i>N</i> -diethandiol- <i>p</i> -toluidine, CQ, water Adhesive: HEMA, MDP, hydrophilic dimethacrylate, <i>N</i> , <i>N</i> -diethandiol- <i>p</i> -toluidine, CQ, silanized colloidal silica, BisGMA	Primer: 1.9 Adhesive: 2.8	00195A 00193A
Clearfil Protect Bond (Kuraray Noritake Dental, Japan)	Apply primer for 20 s. Air gently, apply the bonding resin, light curing for 10 s	Primer: HEMA, MDP, hydrophilic dimethacrylate, MDPB, water Adhesive: HEMA, MDP, hydrophilic dimethacrylate, <i>N</i> , <i>N</i> -diethandiol- <i>p</i> -toluidine, CQ, silanized colloidal silica	Primer: 1.9 Adhesive: 2.8	0012A 0020A
BisGMA: Bisphenol A glycidyl dimethacrylate, CQ: D,1-camphorquinone, HEMA: 2-Hydroxyethyl methacrylate, MDP: 10-Methacryloyloxydecyl dihydrogen phosphate, MDPB: 12-Methacryloyloxy-dodecyl pyridinium bromide				

paraffin. Serial sections of 7 µm thick were prepared from each tooth using a microtome (Leica 2125 RT) and bacterial staining was done with modified Brown and Brenn Gram Stain. Finally, 20 serial sections from each tooth were evaluated under a light microscope (Eclipse E400; Nikon, Kanagawa, Japan) (100 × and 200 × magnification) 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 the cavity walls and floor, and 2- positive bacterial staining within the cut dentin tubules. Ordinal data were statistically analyzed using Kruskal-Wallis and the Mann-Whitney U-tests.

Dye penetration test

As described in the bacterial penetration test, the cavities were prepared, sterilized, and restored with two dentin bonding systems and a composite resin on the buccal and lingual surfaces of 16 extracted wisdom teeth. After thermocycling, the specimens were immersed in 0.5% basic fuchsin for 24 h. The specimens were then sectioned into two parts bucco-lingually with a diamond disk. Each section of the specimen was observed under a stereomicroscope (Olympus SZ 40, Tokyo, Japan) and evaluated using the highest microleakage score from the two parts of each specimen. The scoring was as described in Figure 1.

The data were statistically analyzed using Kruskal-Wallis and the Mann-Whitney U-tests.

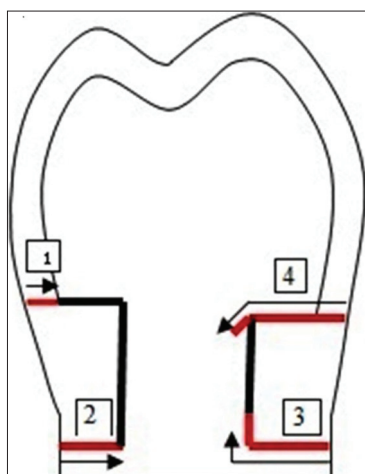


Figure 1: Diagram of microleakage scoring system: 0- no microleakage, 1- dye penetration until the enamel–dentin junction, 2- dye penetration in the cavity walls, 3- dye penetration in the cavity floor, 4- dye penetration partly or completely toward the pulp along dentin

RESULTS

Bacterial penetration test

The results of the bacterial microleakage of the two materials are shown in Table 2. The bacterial stain was detected in some cavities at the cavity wall and floor of both adhesive groups. Additionally, in all control cavities and a few restored cavities, bacterial staining was also observed within the cut dentinal tubules [Figures 2 and 3]. There was no statistically significant difference observed between the bacterial penetrations of the two bonding systems ($P > 0.05$).

Dye penetration test

The results of dye leakage testing for the two materials are shown in Table 3. Dye staining was observed in the enamel margins of three teeth for both bonding systems and in both the enamel and cement margins of one tooth for the CPB group [Figure 4]. This microleakage was scored as 1. There were no statistically significant differences observed between the dye penetration of the CSE and CPB groups ($P > 0.05$). Although more dye leakage was observed in the enamel margins than cement margins for both bonding systems, these differences were not statistically significant either ($P > 0.05$).

DISCUSSION

Some factors, such as pH, viscosity, diffusion capacity, antibacterial agents, and content, may influence the antibacterial action of the adhesive systems.^[7-10] It has also been considered that the acidic primer of self-etching adhesive systems is related to bacterial inhibition.^[9-11] CPB and CSE contain 10-methacryloxydecyl dihydrogen phosphate (MDP) as an acidic adhesion-promoting

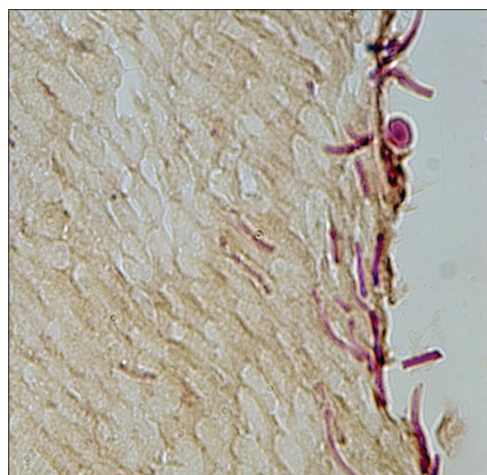


Figure 2: The cavity without restoration in the control group; bacteria were observed in the cavity floor and within the dentin tubules (modified Brown and Brenn ×1000)

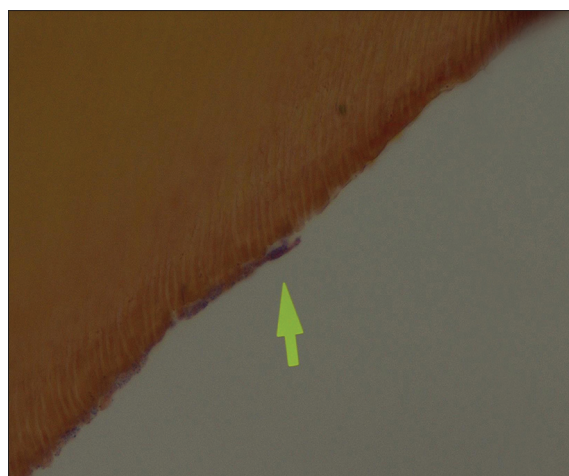


Figure 3: The cavity restored with CPB; bacteria were observed in the cavity walls (modified Brown and Brenn $\times 400$)

Table 2: The results of dye leakage

Groups	n	0	1	2
Clearfil protect bond	16	10	5	1
Clearfil SE bond	16	9	5	2
Control	4	0	0	4

Table 3: The results of bacterial leakage

Groups	Location of margin	n	0	1	2	3	4
Clearfil protect bond	Enamel	16	13	3	0	0	0
	Cement	16	15	1	0	0	0
Clearfil SE bond	Enamel	16	13	3	0	0	0
	Cement	16	16	0	0	0	0

monomer. MDP with a pH value of 2.0 has an inhibitory effect on microorganisms;^[12] however, this inhibitory action should be considered as “limited.” This is because the MDP released from the primer is neutralized by the buffering capacity of the dissolved calcium and phosphate ions from the tooth tissue.^[13] Additionally, the polymerization of adhesive materials decreases the release of acidic monomers and polymerizable antibacterial components;^[9] therefore, the antibacterial activity of adhesive systems is reduced after light activation.

The CPB primer also contains MDPB, in addition to MDP. MDPB confers bacteriostatic properties that help in inhibiting bacterial contact,^[14] and the incorporation of this antibacterial agent into a dentine adhesive system results in strong antibacterial activity against oral streptococci in vitro.^[15,16]

Imazato *et al.* compared the antibacterial potential of the primers before and after the addition of MDPB to the primer's composition, in order to assess the pure



Figure 4: The cavity restored with CPB; dye leakage (score 1) was observed in the enamel and cement margins

contribution of this resin monomer.^[16] The authors observed that in the absence of MDPB, the primer did not exert antibacterial effects against *S. mutans* and *Lactobacillus*, and this finding demonstrates that the antibacterial effect of CPB depends mainly on the presence of MDPB.^[17,18] In another study by Gondim *et al.*, it was determined that CPB exhibited greater antibacterial activity than CSE when applied to paper disks.^[19]

In the present study, the bacterial penetration of restored cavities with CSE and CPB was compared using bacterial staining techniques on the histological sections. Additionally, the marginal sealing capacities of both adhesive systems were determined using dye leakage tests to distinguish the effects of the antibacterial activity from the effect of marginal sealing of the bonding systems on bacterial penetration. The dye penetration test is too sensitive for the determination of microleakage because the dye particles are smaller ($0.12\ \mu\text{m}$) than the size of a typical bacterium ($0.5\text{--}1\ \mu\text{m}$) or the internal diameter of dentinal tubules ($1\text{--}4\ \mu\text{m}$).^[5,20]

S. mutans was chosen as the test microorganism because it is related to dental caries.^[8] These bacteria are small ($0.5\text{--}1\ \mu\text{m}$), allowing for the rapid and easy penetration into the dentin tubules through microgaps,^[21] which can lead to pulp damage.

In previous studies, it has been reported that autoclave sterilization did not affect the bond strengths of some dentin bonding systems;^[22–24] therefore, an autoclave was used in this study to sterilize the teeth.

The teeth were stored in a broth culture of 1.56×10^8 CFU/ml of *S. mutans* at 37°C for 10 days. The bacterial stain was detected at the cavity walls and

floors of five cavities in both groups. Additionally, it was observed within the cut dentin in one cavity from the CPB group, in two cavities from the CSE group, and in all control cavities.

There are very few *in vitro* studies that have investigated bacterial microleakage on the histological sections of extracted teeth.^[21] Usually, *in vivo* and animal studies are used to assess the relationship between the bacteria in the tooth-restoration interface and pulp reactions. The bacteria are investigated by staining the histological sections.^[25-27] In this context, in a direct pulp capping study performed with two self-etching systems in human teeth, Accorinte *et al.* found no bacteria along the cavity wall after 30 days, but there was bacterial staining at the cavity wall in two of six teeth at 90 days for the CSE.^[28] In another study performed in beagles, the bacteria were not observed at the cavity wall following direct pulp capping with CSE at 30 or 90 days.^[29]

In the present study, dye staining was observed in the enamel margins of three teeth for both bonding systems, and in the cement margin of one tooth for the CPB group. These microleakages have been scored as 1. According to these results, the sealing ability of the self-etching adhesives used in the present study is more effective in the cement margins than in the enamel margins, although there was no a statistically significant difference. Carvalho *et al.* speculated that the conditioning efficacy and the penetration of the self-etching adhesive systems in the enamel and dentin depend on the initial acidity of the material and the buffering capacity offered by the substrate.^[30] It is thus expected that these materials have less effectiveness on the enamel due to its higher calcium content.^[30]

The incisal parts of Class V cavities contain more composite material than the cervical parts. Therefore, the bonding between the tooth and restoration more adversely affects the incisal margin, because more stress occurs in this area due to polymerization shrinkage and thermocycling. Kubo *et al.* demonstrated the deterioration in the integrity of the enamel margins due to thermocycling and more leakage in the enamel margins than the cement margins for CPB and CSE.^[31] These results are compatible with ours.

In the present study, statistically significant differences were not observed between the dye leakage of the bonding systems and between the dye leakage of the enamel and cement margins. This finding is consistent with the results of Kubo *et al.* and Siso *et al.*,

which also reported that the CPB and CSE have good sealing ability.^[31,32] In some studies, the CSE and CPB produced similar bonding interfaces.^[33,34]

It is interesting that bacterial stains were seen in the cavity floors and within the dentin tubules, whereas the dye staining was seen in the external cavity walls. Since the size of the bacteria is larger than the size of the dye particles, CPB, especially, has an antibacterial effect. This result may be due to the bacterial contamination of the cavity during preparation or restoration. The bacterial staining technique does not differentiate microorganism viability; therefore, dead bacteria (by autoclave or the antibacterial effect of the bonding system) could also be stained. These results may also be related to the fact that the teeth were stored for 24 h in the dye, but for 10 days in the bacterial samples.

CONCLUSION

Within the limitations of this *in vitro* study, it could not be demonstrated that there was an advantage of MDPB-containing CPB versus CSE in preventing bacterial leakage. Our hypothesis was confirmed. This may be due to the good marginal sealing provided by both bonding systems. The antibacterial monomer containing CPB may be more effective than the one containing CSE for the prevention of bacterial microleakage through the gaps formed by the degradations at the bonding interface caused by aging or trauma. Therefore, further research is needed in order to evaluate the long-term antibacterial effects of MDPB.

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Source of Support: Nil.
Conflict of Interest: None declared