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Bacterial leakage in root canals filled with AH Plus and dentine bonding agents

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Abstract

Objective. The aim of this study was to compare the efficacy of different dentine adhesives in delaying the coronal bacterial leakage of *Enterococcus faecalis* in filled root canals. ***Materials and methods.*** Ninety-five lower incisors of patients >65 years of age were instrumented using the ProTaper® system and were irrigated with 1 mL of 2.5% sodium hypochlorite (NaOCl) alternated with 1 mL 17% EDTA between each file change. Final irrigation was performed with 5 mL of 17% EDTA and then flushed with 5 mL of distilled water. The teeth were randomly divided into five experimental groups ($n = 15$/group) and one of the following dentine adhesives was applied: (1) AdheSE®; (2) Excite® DSC; (3) Clearfil™ Protect Bond; (4) One Coat 7.0; or (5) Control group without adhesive. After filling the root canals, the samples were mounted on a double chamber device to evaluate the bacterial filtration of *E. faecalis* during a period of 240 days. The results underwent non-parametric Kaplan-Meier survival analysis and comparisons among groups were done using the Log-Rank test. ***Results.*** At 240 days, *E. faecalis* was detected in samples of all groups in the lower chamber. The highest survival value was obtained by One Coat 7.0, giving statistically significant differences from the other groups, whereas Clearfil™ Protect Bond, AdheSE® and Excite® DSC showed similar behaviours, likewise similar to the Control group. ***Conclusions.*** One Coat 7.0 adhesive system provides the longest survival value to delay *E. faecalis* coronal leakage in filled root canals.

Key Words: *adhesive systems, antibacterial agents, leakage, Enterococcus faecalis, root canal filling*

Introduction

Successful root canal therapy requires chemomechanical preparation and a complete obturation of the root canal system with non-irritating materials. However, despite the efforts of clinicians, it has been shown that root canal filling materials leak, regardless of the different techniques used [1–4]. Many factors may cause coronal leakage, such as loss of the temporary restoration, breakage of the dental structure, marginal leakage of the final restoration material or recurrent caries. Exposure of the sealed root canal to the oral cavity can lead to recontamination of the root canal [5]. Afterwards, periapical contamination usually occurs within a short period of time [6].

To evaluate the sealing ability of root filling materials, bacterial leakage tests are frequently used [7]. Nonetheless, they must be designed to avoid possible faults that would introduce bias in the results. Some errors have been identified and valid solutions have been proposed to remedy them [8,9].

Dentine bonding agents are widely used in restorative dentistry to improve the bond of materials and to prevent or delay microleakage. In endodontics, these agents have been evaluated in root canal obturation, for perforation repairs, and as root-end barriers [10–12]. Advances in adhesive technology have reinforced the search to minimize both apical and coronal marginal leakage by improving sealer adhesion to root canal walls [13] and, thus, reduce the susceptibility of root-filled teeth to fracture [14]. Research into the use of adhesives in root canal treatment [11,15,16] has shown that microfiltration is reduced when a total-etch [5,17] or self-etching adhesive

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system [18] is used in combination with an epoxy resin or dimethacrylate sealer. In contrast, other studies have found that the use of adhesives does not improve the sealed apical root canal [19] or improve the adhesion of resin-based sealers to dentine [20].

Because dentine adhesive systems may come into direct contact with the residual bacteria in root canal dentine walls, the antimicrobial potential of dentine adhesives is an important consideration. To provide resin-based materials with antibacterial activity, the monomer 12-methacryloyloxydode-cylpyridiniumbromide (MDPB) was developed. MDPB is a compound of a quaternary ammonium antibacterial agent with a methacryloyl group and it exhibits strong antibacterial activity against oral streptococci [21]. The incorporation of MDPB has been reported to be effective in providing dentine bonding systems with antibacterial activity before and after curing [22,23]. Moreover, dentine adhesives with antimicrobial molecules have shown good adhesive results in cavities [24,25]. In this sense, the use of adhesive systems with residual antibacterial activity could provide a supplementary benefit contributing to the delay of bacterial microleakage.

AH Plus® is an epoxy resin-based sealer that has good sealing ability and is considered the referent of comparison for new root canal sealers. When compared with different endodontic sealers, AH Plus® showed the lowest value in terms of both bacterial leakage [26,27] and fluid filtration [28]. However, AH Plus® showed less resistance to bacterial leakage when compared with sealers that are used with adhesive primer [29] or are similar to a self-etch methacrylate resin-based sealer [30].

To our knowledge, no studies have determined the effect of using adhesives with antimicrobial agents prior to root canal filling with an epoxy resin sealer. The aim of this study was to compare, over time, the coronal leakage of *Enterococcus faecalis* in root canals filled with different dentine adhesives and AH Plus® using the cold lateral compaction technique.

Materials and methods

Sample preparation

Ninety-five freshly extracted mandibular incisor teeth with single, straight root canals and fully developed apices, extracted for periodontal reasons, were obtained for the study. The teeth were from patients >65 years old who had 2–4 teeth extracted. They were randomly divided into five experimental groups of 15 each and the remaining 20 specimens were used as positive and negative controls (10 + 10).

Any excess calculus or soft tissue of the teeth was removed using a curette; they were then stored in 0.1% thymol solution at 4°C until use. The teeth were sectioned perpendicular to the long axis using a turbine handpiece and a diamond bur, except the 10 that served as negative controls. To ensure uniformity in the samples, the crowns were cut so as to obtain a root length of ~12 mm from the coronal surface to the apex of the root.

All the specimens were prepared by the same operator. A 10 K file (Dentsply Maillefer, Ballaigues, Switzerland) was placed in the canal until the tip was just visible at the apical foramen and pulled back 1 mm to determine the working length. Instrumentation was performed using ProTaper® instruments (Dentsply Maillefer) with the crown-down technique, and the root canals were enlarged up to an F3 file (finishing file number 3; taper 0.09–0.05; size 30). A size 10 K file was used between each ProTaper® instrument to verify the permeability of the canals. Irrigation was carried out using a 3 mL Luer-Loc syringe coupled to a 30-gauge needle tip placed passively into the canal up to 2 mm from the apical foramen without binding. Alternating irrigation of 1 mL of 2.5% NaOCl and 1 mL of 17% EDTA solutions was carried out between each file. After root canal preparation, the teeth were flushed with 5 mL of 17% EDTA, flushed again with 5 mL of distilled water and then dried with F3 paper points (Dentsply Maillefer).

Adhesive application

All the adhesives were applied according to the manufacturer's instructions. Briefly,

- Group 1-AdheSE® (Ivoclar Vivadent, Schaan, Liechtenstein): the primer was applied with a paper point with continuous rubbing for 30 s. Bonding and Activator were mixed 1:1 and applied into the root canal. Excess of primer and bonding material was removed with F3 paper points.
- Group 2-Excite® DSC (Ivoclar Vivadent): the root canals were etched, washed and dried with F3 paper points before applying the adhesive during 10 s.
- Group 3-Clearfil™ Protect Bond (Kuraray, Osaka, Japan): the self-etching primer was applied with an F3 paper point, left in place for 20 s and dried; afterwards, the bonding agent was applied dried and coronal-light-cured for 10 s.
- Group 4-One Coat 7.0 (Coltène/Whaledent, Langenau, Germany):the Bond and Activator were mixed 1:1 and applied into the root canals using an F3 paper point. The excess was dried.
- Group 5-Control group: no dentine adhesive application was performed.

Root canals were filled using an F3 master gutta-percha point and AH Plus® sealer (Dentsply Maillefer). Lateral condensation was performed using a #25 spreader (Dentsply Maillefer). The gap left by the spreader was filled with accessory gutta-percha points. Excess gutta-percha was cut off using a hot

instrument and the coronal portion of warm gutta-percha was firmly condensed vertically. All these samples were stored at 37°C for 72 h to allow the sealer to set. Ten positive controls were instrumented but not obturated.

Microbial leakage test

The external surfaces of all teeth, excepting 2 mm around the apical foramen and the coronal surface to ~0.5 mm around the root filling material, were covered with two layers of nail varnish in order to prevent bacterial leakage through the lateral canals. Negative controls were fully covered by two layers of nail varnish.

The microbial test consisted of a two-chamber method. The tapered ends of 1.5 mL Eppendorf plastic tubes (Elkay, Shrewbury, MA) were cut and the roots were inserted individually into the tubes until the roots protruded through the end. The interface between the tooth and the Eppendorf tube was put into the rubber cork of a penicillin bottle previously cut to fit inside the lower chamber. The junctions between the root, the Eppendorf and the rubber cork were sealed with cyanoacrylate adhesive (Super Glue-3, Henkel Ibérica, S.A., Barcelona, Spain). The mounts were sterilized for 45 min in hydrogen peroxide gas plasma (Sterrad-50, Johnson & Johnson, Irvine, CA).

After sterilization, the mounts were placed in the glass flasks of the lower chambers, containing 10 mL of sterile Brain Heart Infusion broth (BHI, Scharlau Chemie S.A., Barcelona, Spain), so that at least 3 mm of each root apex were immersed in the broth. The junctions between the Eppendorf tubes and the glass flasks were tightly sealed with Parafilm M™ (Pechiney Plastic Packaging, Chicago, IL) and cyanoacrylate adhesive.

The upper segments of each split chamber were filled with 1 mL BHI broth containing 6×10^8 CFU/mL of *E. faecalis* (ATCC 29212), keeping the bacterial suspension in contact with the coronal portion of the filled roots. The mounts were always handled in sterile conditions under a laminar flow hood (Nuaire, Plymouth, MN) to avoid bacterial contamination. The whole system was incubated at 37°C during a period of 240 days. Twice weekly the medium in the upper chamber was replaced with freshly grown broth and the viability of the bacteria was checked every week by seeding it in agar blood plates.

The lower chambers of all mounts were observed daily and turbidity time was recorded for each specimen as an indicator of entire root canal contamination. Once turbidity was present, a sample of the turbid broth was streaked onto blood agar plates and bacteria were identified to ensure that there was no contamination other than *E. faecalis*.

Statistical analysis

The proportion of unleaked samples over 240 days was evaluated using non-parametric Kaplan-Meier survival analysis. In this study the term survival is used with the understanding that a sample survives when it does not leak at a given time. The overall evolution of leaked *E. faecalis* in the samples was analysed, taking into account the entire time period, not just at one or more intervals in time. Differences among groups were analysed using the Log-Rank test at a significance level of 0.05. All statistical analyses were performed by means of SPSS 17.0 software (SPSS Inc., Chicago, IL).

Results

All positive controls exhibited bacterial leakage within 48 h, whereas the negative controls remained unleaked throughout the study. The number of leaked samples and the median day of bacterial leakage are given in Table I. At 240 days, *E. faecalis* was detected in all samples in the lower chamber of the groups AdheSE®, Excite® DSC and the Control, as well as in 14/15 of Clearfil™ Protect Bond and 11/15 of One Coat 7.0.

The results of Kaplan-Meier survival analysis are shown in Figure 1. Pair comparisons determined by the Log-Rank test show the highest survival value for One Coat 7.0, which gave statistically significant differences with respect to the other groups. It was followed by Clearfil™ Protect Bond, AdheSE® and Excite® DSC; there were no significant differences among these, or with regard to the Control group.

Table I. Number of leaked samples after 240 days (n = 15 per group).

Groups	Leaked samples	Minimum	Maximum	Median
AdheSE®[a]	15 (100%)	35	177	113
Excite® DSC[a]	15 (100%)	42	240	87
Clearfil™ Protect Bond[a]	14 (93.3%)	42	>240	90
One Coat 7.0[b]	11 (73.3%)	64	>240	153
Control[a]	15 (100%)	65	213	110

The same superscript letter shows differences that were not statistically significant as determined by the log-rank test ($p < 0.05$).

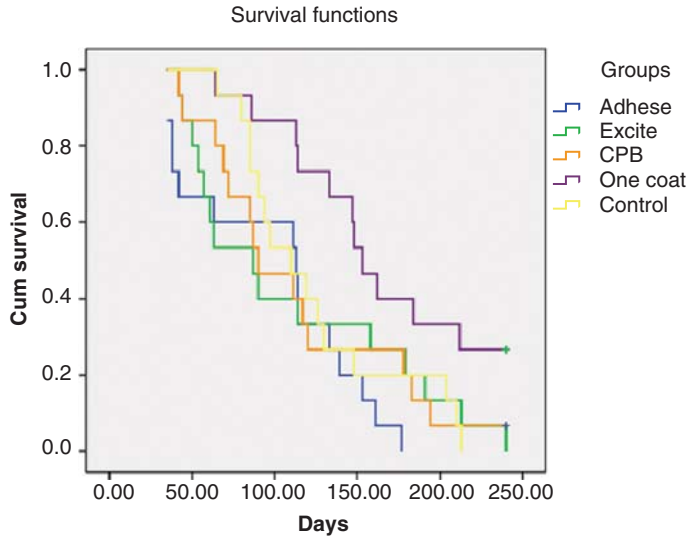

Figure 1. Kaplan Meier survival probability at 240 days (probability of no leakage) for all groups. Cum survival: percentage of samples that did not show *E. faecalis* leakage at a given time. X-axis: Days of incubation. Y-axis: Proportion of roots resisting leakage.

Discussion

This study evaluated whether the use of dentine adhesives with and without antimicrobial activity could slow the coronal leakage of *E. faecalis* in the root canal filling. Several methodologies for the assessment of apical and coronal leakage of endodontic filling materials have been described, although their validity has been questioned [7–9]. The use of bacteria as markers in an *ex vivo* model was introduced to overcome some of the limitations of dye leakage studies. Possible methodological errors have recently been identified in the context of two-chamber bacterial filtration analysis [8,9]. In this study, to overcome such limitations, we used a greater number of positive and negative controls ($n = 10$) and, instead of using wax, the interface of the coronal and apical chambers was sealed with a rubber stopper and cyanoacrylate; this measure proved effective, as none of the negative controls (intact crown and upper chamber with bacteria) passed bacteria to the lower chamber. Furthermore, the dentine tubules were sealed by painting with nail varnish the outer coronal surface—resulting from cutting—exposed to the bacteria, leaving only ~0.5 mm around the root filling material. In this way we eliminated potential routes of leakage other than the root canal, ensuring greater validity of the methodology. The follow-up time of 240 days can be considered long, in light of similar studies [3,27], but many samples in all the groups still showed no leakage. Finally, we used survival analysis to determine the overall evolution of leakage, taking into account the entire time period and not just one or more points in time.

In this study, 5 mL of 17% EDTA was used as the final irrigation solution, followed by 5 mL of distilled water. Generally, the use of 17% EDTA followed by 5.25% NaOCl is an effective protocol for removal of smear layer from canal walls and dentinal tubules [31]. However, NaOCl is a strong oxidizing agent and leaves behind an oxygen-rich layer on the dentine surface, which results in reduced bond strengths [32] by inhibiting the polymerization of resins [33] and increased microleakage [34].

The adhesives selected included Excite® DSC, a total-etch adhesive; AdheSE®, a self-etching adhesive, Clearfil™ Protect Bond, with a known antimicrobial molecule, and One Coat 7.0, whose antimicrobial component is not indicated by the manufacturer. Although the dual-cure adhesive system ensures better polymerization in the deeper region of the root canal system, a light cure adhesive, Clearfil™ Protect Bond, was tested in this study because it has been shown to effectively reduce the surface attachment of some strains of bacteria such as *Streptococcus mutans* [35].

The results of this study demonstrate delayed coronal leakage over time when using One Coat 7.0, which gave statistically significant differences with respect to all the study groups. It is noteworthy that it took over 150 days for half the specimens to leak (median = 153) and that, at 240 days, there were four samples that still showed no microleakage. This substantial delay in coronal filtration may largely be due to the antibacterial molecule present in the formulation of One Coat 7.0, as well as to the smaller degree of conversion in the one-step adhesives [36], allowing the antimicrobial molecule to act freely and with greater efficacy for a longer time.

Clearfil™ Protect Bond has exhibited antibacterial efficacy against *E. faecalis* when evaluated by a direct contact test [37] and it exerts, even without polymerization [23], a bactericidal effect against *S. mutans*,

both in planktonic cultures and in biofilms, with a short exposure period (20 s). In the case of our study, global results proved similar to those of the Control group and the rest of the adhesives used. As a photopolymerizable adhesive, the possibility of curing inside of the root canal would be limited to the first millimetres of the root canal. This might facilitate the entry of micro-organisms, to be compensated precisely by antimicrobial activity. Er et al. [16], using a bacterial filtration system in root end cavities, where it is possible to achieve better polymerization, observed a statistically similar behaviour for Clearfil™ Protect Bond and other adhesives, although their follow-up period was short (4 weeks) and gave fewer leaked samples (2/15).

Excite® DSC, an 'etch and rinse' adhesive with good adhesion to dentine [38], effectively inhibits the biofilm formation of *E. faecalis* on its surface [37] and could be considered *a priori* a dentine adhesive useful in filling root canals. Also, given its hydrophilic properties, it might have an additional advantage in slightly moist canals [17]. In the present study, however, the application of Excite® DSC did not substantially delay the coronal leakage of *E. faecalis*, and it had a behaviour similar to those of Clearfil™ Protect Bond, AdheSE® and the Control group. In fact, this adhesive was seen to allow the earliest leakage, in 50% of the samples (median = 87).

When the bactericidal capacity of AdheSE® was evaluated, it was found to be effective against *S. mutans*, *Lactobacillus casei* and *Lactobacillus acidophilus* [39] using the disk diffusion method. Two-step dentine adhesives have shown better results than other adhesive systems in terms of resistance to hydrolytic degradation [40]. Meanwhile, self-etch adhesive systems have advantages over the total-etch systems, involving moisture content when applied in dentine, and the interaction of monomers with the dentine that diffuse to the depth where the surface has demineralized, promoting interlocking [41]. In the survival analysis of our study, however, AdheSE® had results similar to Clearfil™ Protect Bond and Excite® DSC. It is possible that the inherent advantages of the composition/mechanism of action of the adhesives Excite® DSC and AdheSE® in conjunction with Clearfil™ Protect Bond were counteracted by the antimicrobial molecule present only in Clearfil™ Protect Bond, so that the results of all three proved similar.

The Control group displayed different short-term and long-term effects. In the first 100 days, its survival behaviour resembled that of One Coat 7.0, but then it began to show faster leakage, overtaking the other groups. The positive findings with the Control group could be related with the use of an epoxy resin, AH Plus®. The application of a decalcifying agent before the use of AH Plus® improved bond strength and sealing ability of this epoxy resin root canal sealer [42], which may have reduced leakage. Furthermore, a recent study shows that AH Plus® and Gutta Flow have similar levels of sealing ability, regardless of the filling technique used [43]. Nonetheless, Ricucci and Bergenholtz [44] report that well-prepared and well-filled root canals resist bacterial penetration, despite oral exposure through caries, fracture or loss of restoration for a prolonged period.

The results of leakage tests are not always consistent with direct contact test methods to test antimicrobial or antibiofilm activity. Bearing in mind that the former are closer to clinical reality and that clinical studies are scarce, bacterial leakage studies could be considered a significant part of endodontic research to compare the capacity of different materials. This methodology is more sensitive than a histological study because, in theory, if just one viable microorganism reaches the lower chamber, turbidity can appear [8]. In addition, it provides data that are more biologically significant and clinically relevant [7].

Within the limitations of this *in vitro* study, One Coat 7.0 showed greater effectiveness in delaying coronal leakage, although none of the adhesives or the Control group completely prevented bacterial leakage during the entire period.

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