Antibacterial Activity of Endodontic Sealers by Modified Direct Contact Test Against *Enterococcus faecalis*

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Abstract

Introduction: The antibacterial effectiveness of 7 different endodontic sealers, AH Plus, Apexit Plus, iRoot SP, Tubli Seal, Sealapex, Epiphany SE, and EndoRez against *Enterococcus faecalis* was studied in vitro. **Methods:** A modified direct contact test was used. Bacteria in suspension were exposed to the materials for 2–60 minutes by using sealers that were freshly mixed or set for 1, 3, and 7 days. The pH values and contact angles of sterile water on sealers at different times after setting were also measured. **Results:** Fresh iRoot SP killed all bacteria in 2 minutes, AH Plus in 5 minutes, EndoRez in 20 minutes, and Sealapex and Epiphany in 60 minutes. Freshly mixed Apexit Plus and Tubli Seal failed to kill all bacteria at 60 minutes. For 1-day and 3-day samples, iRoot SP and EndoRez had the strongest antibacterial activity, followed by Sealapex and Epiphany; Tubli Seal and AH Plus did n'ot show any significant antibacterial activity. Of all the samples, Apexit Plus had the lowest antimicrobial activity. The pH of the sealers could not alone explain their antibacterial effect. **Conclusions:** Fresh iRoot SP, AH Plus, and EndoRez killed *E. faecalis* effectively. IRoot SP and EndoRez continued to be effective for 3 and 7 days after mixing. Sealapex and EndoRez were the only ones with antimicrobial activity even at 7 days after mixing. *(J Endod 2009;35:1051–1055)*

Key Words

Antibacterial activity, direct contact test, endodontic sealers, *Enterococcus faecalis*

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Microbes and microbial products are the main etiologic factors of pulpitis and apical periodontitis (1, 2). Therefore, an important aim of endodontic therapy is the elimination of microorganisms from the root canal. Instrumentation, irrigation, and intracanal medication significantly reduce the population of microorganisms inside the infected root canal. It is impossible, however, to completely eliminate the microbes from the root canal system in all cases. Consequently, the use of root canal filling materials with antibacterial activity is considered beneficial in the effort to further reduce the number of remaining microorganisms and to eradicate the infection.

Many studies have been performed to assess the antimicrobial activity of different endodontic sealers (3–8). There is little or no information available about the antimicrobial properties of iRoot SP (Innovative Bioceramix, Vancouver, Canada; also known as EndoSequence BC sealer, Brasseler, Savannah, GA) and Resilon/Epiphany (Pentron, Wallingford, CT), 2 new endodontic sealers on the market (3, 4).

The agar diffusion test (ADT) used to be the most commonly applied method to assess the antimicrobial activity of endodontic sealers (5–8). However, the limitations of this method are nowadays well-recognized. The results obtained are not likely to reflect the true antimicrobial potential of the various sealers or disinfecting agents; therefore, ADT is no longer recommended to be used for this purpose in endodontic research (9, 10). A direct contact test (DCT), which circumvents many of the problems of ADT, was first introduced by Weiss et al (11, 12) for the evaluation of the antimicrobial effect of endodontic sealers and root-end filling materials. The test is a quantitative and reproducible assay that allows testing of insoluble materials and can be used in standardized settings.

Enterococcus faecalis, the most frequently recovered microorganisms from refractory periapical periodontitis (13), has been used in numerous studies of the antibacterial properties of disinfecting agents because of its resistance to some medicaments and its ability to survive conventional root canal therapy (3–7, 11). In this study, a strain of *E. faecalis* isolated from a case of persistent apical periodontitis (14) was used as a test organism. The purpose of this study was to use a modified DCT assay to evaluate the antibacterial activity of 7 different endodontic sealers against *E. faecalis* 20 minutes after mixing (fresh samples) and 1, 3, and 7 days after mixing (set samples).

Material and Methods

Sealers

Seven endodontic sealers were used in this study: an epoxy resin–based sealer, AH Plus (Dentsply International Inc, York, PA); 2 polymethacrylate resin–based sealers, Epiphany SE (Pentron Clinical Technologies LLC, Wallingford, CT) and EndoRez (Ultradent, South Jordan, UT); 2 calcium hydroxide–based sealers, Apexit Plus (Vivadent, Schaan, Liechtenstein) and Sealapex (SybronEndo Corporation, Orange, CA); a calcium hydroxide–calcium silicate complex sealer, iRoot SP; and a zinc oxide–eugenol–based sealer, Tubli Seal EWT (SybronEndo Corporation, Orange, CA). Epiphany SE and EndoRez were tested as both light-cured and non–light-cured.

Microorganism

Enterococcus faecalis VP3-181, isolated from a case of persistent apical periodontitis (14), was used as a test organism. It was grown overnight in air at 37°C on

Tryptic Soy Agar (TSB; Becton, Spark, MD) plates for the experiments. After checking for purity, *E. faecalis* was suspended in sterile water and adjusted to a density of 3×10^8 colony-forming units (CFU)/mL by using a Microplate Reader model 3550 (BIO-RAD, Hercules, CA) at 405 nm.

Modified DCT

The DCT used to assess the antimicrobial effect of the endodontic sealers has been described earlier in detail (11). In the present study, all sealers were prepared in strict compliance with the manufacturers' instructions. A 96-well microtiter plate (Sarstedt Inc, Newton, NC) was held vertically, and an area of fixed size on the side wall of the wells was coated with an equal amount of each material by using a cavity liner applicator. The sealers tested 20 minutes after mixing were designated as fresh specimens (group 1); other specimens were allowed to set for 1, 3, and 7 days in a humid atmosphere at 37°C before testing (groups 2–4).

A 10 μL of bacterial suspension (3×10^8 CFU/mL, which contained 3×10^6 bacteria) was carefully placed on the surface of each sealer. Bacterial suspensions placed on the wall of uncoated wells were used as control. After incubation in 100% humidity at 37°C for 2, 5, 20, and 60 minutes, 240 μL of TSB was added to each well. After gently mixing with a pipette for 1 minute, the bacterial suspension from each well was transferred and serially diluted in TSB. The survival of bacteria was assessed by culturing aliquots of 20 μL onto TSA plates after 10-fold serial dilutions. After incubation for 24 hours at 37°C, colonies on the plates were counted, and the CFU/mL was calculated. All experiments were performed in triplicate.

Controls for Carryover Effect

To monitor the carryover effect of the sealers, an area of fixed size on the side wall of wells was coated with the same amount of sealer as for DCT. Twenty minutes after mixing, 10 μL of sterile water was placed in direct contact with each specimen. After incubation in 100% humidity at 37°C for 1 hour, TSB (240 μL) was added to each well. After gentle mixing for 1 minute, 10 μL of the broth was transferred into 970 μL TSB. A 20 μL of bacterial suspension (7×10^6 bacteria) was added at the same time to this first dilution tube. In another carryover control, no sealer was used, but the same amount of sterile water (10 μL) was placed on the wall of uncoated wells and processed further as above. The possibility of carryover of the sealers' antibacterial activity was assessed by culturing 10-fold serial dilutions onto TSA plates and by comparing the survival of added bacteria in the 2 carryover controls (with and without sealer). After incubation for 24 hours at 37°C, colonies on the plates were counted, and CFU/mL was calculated. The carryover tests for each sealer were performed in triplicate.

Contact Angle Measurements

Contact angle measurement was used to characterize the wettability of the sealers by sterile water. The sealers were spread evenly onto glass slides, and the samples were kept in 100% humidity at 37°C. Contact angle measurements were conducted 20 minutes, 1 day, 3 days, and 7 days after mixing by placing 10 μL of sterile water on each sealer's surface. Within 30 seconds, the contact angle was measured by using a NRL Contact Angle Goniometer (Ramé-hart, Netcong, NJ).

pH of the Sealers

An equal amount of each sealer was applied to cover half of the bottom surface (98 mm^2) of the wells of 24-well plates and kept in 100% humidity at 37 °C. Twenty minutes, 1 day, 3 days, and 7 days after mixing, 3 mL of sterile water was added to each well. The pH values were measured at 3, 20, and 60 minutes after adding the water by using a temperature-compensated electrode with a pH meter (SB70P; VWR, West Chester, PA).

Effect of Low pH on Bacterial Viability

Bacterial suspension (3×10^8 CFU/mL) was mixed with phosphate buffer at 2 different pH values (3 and 3.5) at 1:24 ratio. Bacteria mixed with sterile water (pH 7) were used as a control group. After incubation at 37°C for 2, 5, 20, and 60 minutes, samples were transferred and serially diluted in TSB before culturing onto TSA plates. After incubation for 24 hours at 37°C, colonies were counted, and CFU/mL was calculated. All experiments were performed in triplicate.

Data Analysis

The mean values of log10 CFU/mL and the standard deviation (SD) of bacteria were calculated. The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparison. The level of significance was set at 95%. Statistical analysis was performed with the statistical software SPSS v. 11.0 (SPSS for Windows; SPSS Inc, Chicago, IL).

Results

The results of the antibacterial effects of the endodontic sealers from modified DCT are presented in Fig. 1. Fresh sealers and sealers set for 1, 3, and 7 days showed differences in their activity against *E. faecalis*. The antibacterial effect of the sealers was relatively stable for up to 3 days. However, after 7 days most sealers had lost much of their antibacterial effect except for Sealapex and EndoRez.

Fresh iRoot SP eradicated all bacteria within 2 minutes of contact. Fresh AH Plus and EndoRez (both light-cured and non–light-cured) significantly reduced ($P < .05$) the numbers of viable bacteria at 2 minutes and killed all bacteria within 5–20 minutes. All other sealers, when freshly mixed, required a minimum of 20 minutes to start killing the bacteria in significant numbers. Despite a reduction in bacterial counts, Apexit Plus, Tubli Seal, and light-cured Epiphany failed to eradicate all bacteria during the 60 minutes of contact with fresh sealers (Fig. 1*a*).

After 1 day of setting, iRoot SP and EndoRez reduced the number of bacteria significantly during the first 2 minutes of contact ($P < .05$), and all bacteria were killed within 20 minutes. Sealapex killed the bacteria at 60 minutes of contact, whereas the other sealers, including AH Plus, failed to kill all bacteria during the 60 minutes of challenge. The results of sealers set for 3 days were similar to those set for 1 day.

Seven days after mixing, EndoRez and Sealapex showed the strongest antibacterial activity, killing all *E. faecalis* cells at 20 and 60 minutes, respectively. Only slight or no antibacterial activity was registered with all the other sealers at this point.

The contact angles of sterile water on sealers at different time intervals after setting are presented in Table 1. IRoot SP showed by far the lowest contact angle, less than 5 degrees after setting. The contact angle of Epiphany and EndoRez decreased from 50 to 35 degrees during setting. Fresh Tubli Seal had a lower contact angle than AH Plus, Apexit Plus, and Sealapex. However, after setting, all 4 sealers had similar high contact angles of 75–90 degrees.

The pH values of the sealers at different times after mixing are shown in Table 2. IRoot SP had the highest pH value (10.7–12.0) in all groups. Apexit Plus and Sealapex also showed alkaline pH values, which increased slightly with increasing setting time. The pH of AH Plus was alkaline only in the fresh sample, whereas after setting, the pH was close to neutral. Tubli Seal had neutral pH values in all groups.

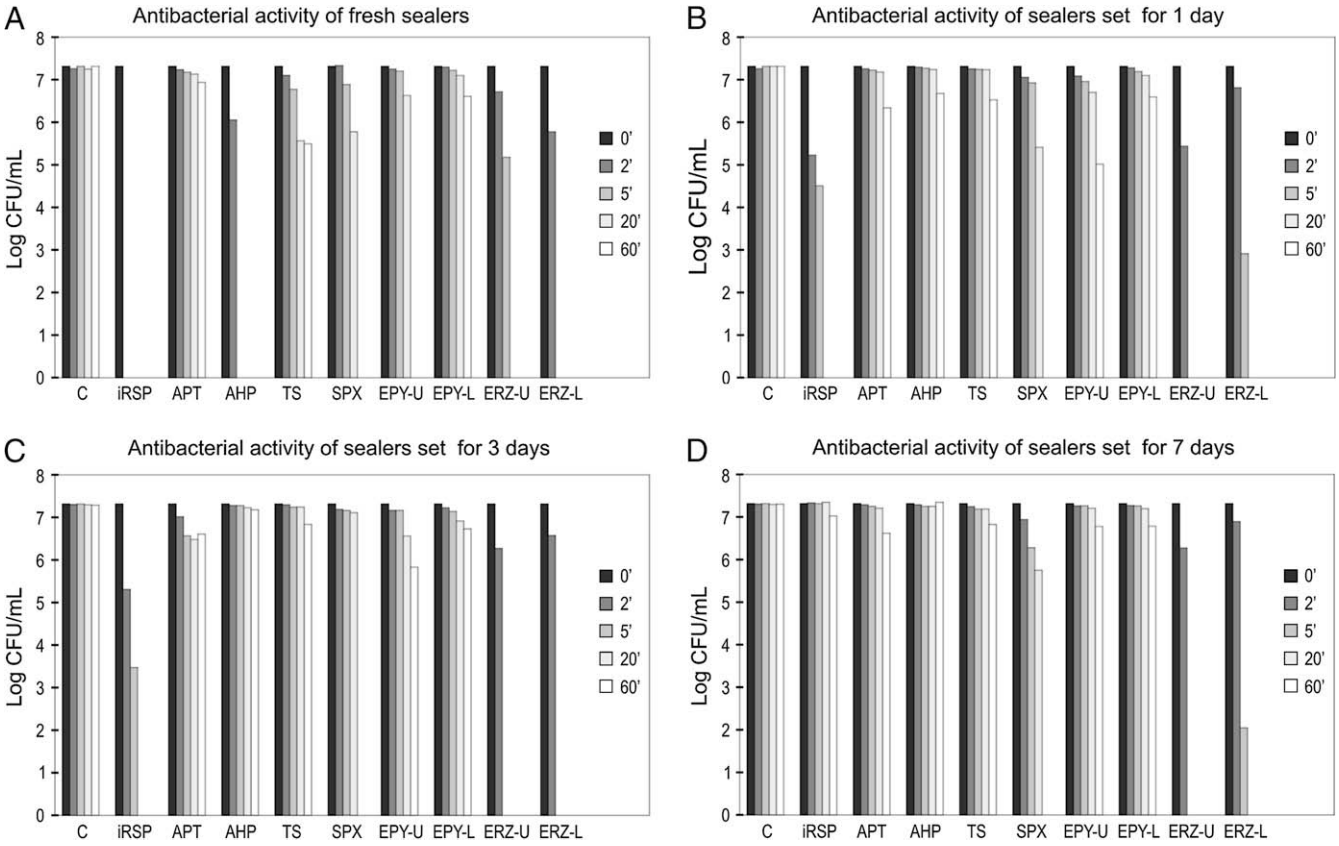

Figure 1. Survival of *E. faecalis* strain VP3-181 after direct contact with sealers for 2, 5, 20, and 60 minutes. (*A*) Fresh sealers, (*B*) sealers set for 1 day, (*C*) sealers set for 3 days, (*D*) sealers set for 7 days. C, Control; iRSP, iRoot SP; APT, Apexit Plus; AHP, AH Plus; TS, Tubli Seal; SPX, Sealapex; EPY-U, Epiphany non–light-cured; EPY-L, Epiphany light-cured; ERZ-U, EndoRez non–light-cured; ERZ-L, EndoRez light-cured.

Epiphany and EndoRez showed acidic pH values throughout the study period.

Control experiments showed that there was no carryover of the antibacterial effect of any of the sealers to the bacterial cultures. Incubation of the bacteria in buffer at low pH did not cause any reduction in viable counts.

Discussion

An ideal endodontic sealer should be biocompatible and dimensionally stable; it should seal well and have a strong, long-lasting antimicrobial effect (15–17). Antibacterial activity of sealers might help to eliminate residual microorganisms that have survived the chemomechanical instrumentation and thereby improve the success rate of endodontic treatment. One of the challenges in endodontic research has been the lack of standardized in vitro and in vivo protocols for the testing of the antimicrobial effect of sealers.

The DCT is a quantitative and reproducible method that simulates the contact of the test microorganism with endodontic sealers inside the root canal. The effect of sealers at various stages of the setting reaction on microbial viability can be evaluated (11, 12, 18). The method also allows for better control of possible confounding factors than ADT. In DCT, the turbidimetric method allows detecting the prevention of growth (bacteriostatic effect). Also, in cases in which carryover effect is controlled, turbidimetric measurements in DCT can show whether all (100%) bacteria have been killed. In the present study, the DCT method was modified in such a way that plating was done immediately after each time of contact. This modification, together with controls for carryover, makes it possible to measure the bactericidal effect instead of bacteriostatic effect of the materials. It also makes it possible to directly calculate the exact numbers of surviving bacteria after each contact time. In clinical endodontics, the bacteriostatic effect might be regarded as less important because the surviving bacteria can continue growth after removal or loss of activity of the medicament or sealers. Therefore, in the present study, the antimicrobial activities of 7 sealers were evaluated by a modified DCT method for direct evaluation of the bactericidal effect of the sealers. Theoretically, lack of growth on the plates could be a result of bacteria having changed into a so-called viable but nonculturable (VBNC) state because of the stress caused by the antimicrobial components of the sealers. However, development of VBNC bacteria typically requires several days of continuous stress and is therefore unlikely to be a factor in this study (19).

Carryover means that some of the medicament or antibacterial substance is unintentionally "carried over" from the exposure test to

TABLE 1. Mean Contact Angle of Sterile Water on Sealers

Sealer	Fresh	1 Day	3 Days	7 Days
iRootSP	25	<5	<5	<5
Epiphany non–light-cured	50	40	35	35
EndoRez non–light-cured	50	40	37	37
AH Plus	66	80	83	83
Apexit Plus	75	80	80	83
Tubli Seal	45	80	80	83
Sealapex	80	90	88	75

TABLE 2. pH Values of Sealers of Different Times after Setting in Sterile Water at 3, 20, and 60 Minutes

	Time after setting											
	Fresh			1 Day			3 Days			7 Days		
Sealer	3	20	60	3	20	60	3	20	60	3	20	60
iRootSP	10.9	11.2	11.5	11.1	11.5	12.0	10.7	11.5	11.8	10.8	11.7	11.8
Apexit Plus	8.6	10.1	10.2	9.9	10.2	10.2	10.0	10.1	10.2	10.3	10.6	10.6
Sealapex	8.2	9.8	10.6	10.0	10.1	10.3	10.0	10.2	10.5	10.5	10.5	10.5
AH Plus	9.5	10.5	10.6	6.3	6.7	6.9	6.0	6.3	6.9	7.6	7.8	7.5
Tubli Seal	7.3	7.1	6.9	6.1	6.3	6.5	5.9	6.4	6.5	7.2	7.3	7.1
Epiphany non–light-cured	5.2	5.2	4.5	5.1	4.7	4.6	5.6	5.0	4.6	5.7	4.5	4.4
EndoRez non–light-cured	4.0	3.5	3.4	4.4	3.8	3.5	4.6	3.8	3.6	4.2	3.7	3.6

the culture broth or culture plates, inhibiting the growth of those microbes, which in fact survived the direct exposure, creating a false-negative result. The possibility of carryover is greater when substances containing antibiotics are used or when other disinfecting agents are used in high concentrations. It is likely that the risk for carryover effect is low with endodontic sealers. In the present study, a series of control experiments were performed to evaluate the possibility of the carryover effect. No difference in colony counts between positive and negative carryover controls confirmed that no carryover took place in the experiments of the present study. In several DCT studies, the experiments to measure the bacteriostatic effect have been done by adding culture broth to the microtiter well harboring the sealer and the bacteria. This approach does not avoid the risk that the sealer can continue to affect the bacteria in the broth. In the present study, bacterial counts were obtained directly after the indicated times of contact to minimize the effect of confounding factors and to facilitate comparisons between the sealers.

Pizzo et al (20) reported that in DCT only fresh AH Plus possessed antibacterial activity, whereas 24-hour and 7-day-old samples did not show antibacterial effect against *E. faecalis*. Similar results were reported by Kayaoglu et al (21). The antimicrobial effect of epoxy resin–based sealers might be related to the release of formaldehyde during the polymerization process (22). The present study also showed that fresh AH Plus had significant antibacterial effect, whereas set samples did n'ot show antimicrobial activity.

The effectiveness of Sealapex against facultative microorganisms has been studied and reported (5, 7, 8). Heling and Chandler (7), with the dentin block model, reported that Sealapex had greater antibacterial effect at 7 days than at 1 day after mixing. In another study (8), DCT assay indicated that AH Plus is a more potent inhibitor of bacterial growth than Sealapex. Fuss et al (23) investigated the antibacterial activity of 2 calcium hydroxide–containing endodontic sealers, Sealapex and CRCS (Hygenic, Akron, OH), and 1 zinc oxide–eugenol–containing sealer, Roth's cement (Roth International Ltd, Chicago, IL). The results showed that Sealapex was weakest in fresh and 1-day-old samples, whereas in 7-day-old samples it showed the strongest antimicrobial effect. The possible reason is a longer setting time, allowing more hydroxyl ions to be released from Sealapex (24–27). Duarte et al (28) reported that Sealapex presented higher calcium and hydroxyl release than Apexit Plus, especially after longer time intervals of 30 days. The results of the present investigation also showed that Sealapex had consistent antimicrobial activity throughout the study.

Apexit Plus started to show limited antimicrobial effect only after 20 minutes of contact time. Its pH value was identical with Sealapex. The contact angle measurement results showed comparable values for Apexit Plus, AH Plus, Tubli Seal, and Sealapex. In the only previous study with DCT test (3), Apexit Plus showed antibacterial effect up to 1 day after mixing. In the same study, no antibacterial effect was detected with AH Plus and Epiphany SE. However, it should be noted that only bacteriostatic effect was examined in that study, and killing some but not all bacteria might have been left undetected.

IRoot SP is a new endodontic sealer, chemically based on Bioaggregate, a ceramic root-end filling material (29). The present study showed it possessed potent antibacterial effect. The sealer is a complex form of calcium silicate cement, calcium phosphate, and calcium oxide. Moisture from dentin is supposed to facilitate the hydration reactions of calcium silicates to produce calcium silicate hydrogel and calcium hydroxide (30). Calcium hydroxide partially reacts with the phosphate to form hydroxyapatite and water (31). The water is supposed to start again the reaction cycle and react with calcium silicates to produce calcium silicate hydrogel and calcium hydroxide. This might explain the high pH of the sealer during the whole study. IRoot SP is also hydrophilic, as shown by the low contact angle determined. The antibacterial effect of iRoot SP sealer might be a combination of high pH, hydrophilicity, and active calcium hydroxide diffusion. However, the antimicrobial effect was greatly diminished at 7 days after mixing.

Epiphany SE (self-etch) sealer and EndoRez are dual-cure hydrophilic methacrylate resin–based endodontic sealers (32). In previous studies with DCT, EndoRez did n'ot show antibacterial activity (33), and Epiphany SE even enhanced bacterial growth (3). Again, our study measured bactericidal activity, whereas the previous studies used methods to assess bacteriostatic effect (3, 33). EndoRez demonstrated strong antibacterial effect against *E. faecalis* throughout the 7-day testing period, and all bacteria were killed during 5–20 minutes of contact with the sealer. EndoRez was clearly sticky with a moist surface even 7 days after mixing, which indicates that the setting of the sealer was not yet complete at this point. Incubation of *E. faecalis* for 1 hour at pH 3 and 3.5 showed that low pH alone does not have an impact on its viability. Slow setting, elution of nonreacted monomers, and the lowest pH (below 4) are probably important for the continuing antibacterial effect of EndoRez.

Different sealers showed different pH values, which also changed during the incubation. Comparison of the pH values and the effectiveness in killing the test organism, *E. faecalis*, indicate that there are factors other than pH that are more important for their antibacterial activity. Apexit Plus had the same pH as Sealapex at all times, yet Sealapex was superior to Apexit Plus, which failed to kill the bacteria even during the longest time of contact. In addition, whereas iRoot SP continuously showed the highest pH of all sealers, after complete setting (7 days) its ability to kill *E. faecalis* cells was almost absent.

The measurement of contact angle can provide useful information of material wettability (34). The lower the contact angle (wettability), the more hydrophilic the substrates are, and the faster the liquid will spread on substrates and wet the surface (35). Potentially this could

influence the antibacterial effect of endodontic sealers, whereas in the present study, contact angle values did not correlate with the antibacterial effect of the sealers. However, a low contact angle, indicative of hydrophilic surface characteristics of a sealer, could facilitate the penetration of the sealer into the fine details of the root canal system and thereby positively affect their antibacterial effectiveness in vivo.

In conclusion, the results of the present study showed that fresh iRoot SP, AH Plus, and EndoRez killed *E. faecalis* effectively. IRoot SP and EndoRez continued to be effective for 3 and 7 days after mixing, respectively. Sealapex was moderately effective throughout the study and was, together with EndoRez, the only sealer that could eradicate *E. faecalis* during the whole study period.

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