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ORIGINAL ARTICLE

Antibacterial activity of various root canal sealers and root-end filling materials in dentin blocks infected *ex vivo* with *Enterococcus faecalis*

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Abstract

Objective. The aim was to investigate the antibacterial activity of the root-end filling materials MTA and IRM, different endodontic sealers and calcium hydroxide [$Ca(OH)_2$] in experimentally infected dentinal tubules. ***Materials and methods.*** Ninety-four human root segments were prepared and the root canals were enlarged to ISO size 90. After smear removal, the specimens were infected with *Enterococcus faecalis* for 3 weeks. The roots were divided into eight groups and filled either with MTA, IRM, $Ca(OH)_2$, gutta-percha and EndoRez (ER)/GuttaFlow (GF)/AH Plus (AH+) or with Resilon and Epiphany (EpRe). One group of specimens was left unfilled for control. Half of the specimens were treated for 1 day and the other half for 7 days in humid conditions at 37°C. Dentin samples from each canal were collected by enlarging the canals to ISO size 150; thus a dentinal depth of 300 µm was sampled. The number of cultivable bacteria was determined for each specimen. Statistical significance was set to 5%. ***Results.*** After 1-day or 7-days of treatment, compared to control, all materials (except ER and GF at day 7) significantly reduced the number of bacteria. At day 1 and day 7, no significant difference was found between ER and GF and between $Ca(OH)_2$, AH+, EpRe, IRM and MTA. However, a significant difference was found between these two groups of materials (except between GF and EpRe at day 7). Significantly more bacteria were cultured in the ER, GF, EpRe and IRM groups at day 7 compared to day 1. ***Conclusions.*** All materials exerted varying degrees of antibacterial activity which generally tended to decrease with time. The most stable antibacterial effect throughout the 7-day period was for $Ca(OH)_2$, AH+ and MTA.

Key Words: *antibacterial agents, endodontics, root canal filling materials, root canal therapy*

Introduction

Bacteria that infect root canals may survive even after thorough chemomechanical preparation of the root canal system [1] and the dentinal tubules may be a source of recurrent infection after conservative as well as surgical treatment [2].

Bacteria remaining after instrumentation can be eliminated or new bacteria can be prevented from reinfecting the root canal system by an inter-appointment dressing such as calcium hydroxide [$Ca(OH)_2$] [3]. However, $Ca(OH)_2$ does not consistently produce bacteria-free canals, may allow regrowth [4] and is not effective against all bacterial species found in root canal infections [5].

Root filling materials also play a role in endodontic infection control by entombing residual micro-organisms and by their antimicrobial activities [6,7]. This may be important when apical periodontitis is treated with a one-step approach instead of the traditional two-step treatment with inter-appointment disinfection.

The antimicrobial activity of endodontic sealers and root canal filling materials has been previously studied mostly on agar plates [8]. However, the relevance of the results from agar diffusion test is

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questionable as agar diffusion test is not standardized to endodontic materials. A direct contact test assay designed to overcome the limitations of agar diffusion test was described by Weiss et al. [9] Studies using the direct contact test have mostly assessed short term antibacterial activity [10-12].

Different components of dentin can inhibit the antibacterial effect of irrigants and inter-appointment medicaments [13-15]. Similar inhibition may be the case also for endodontic sealers. The dentin block model, used originally for testing the antibacterial activity of root canal disinfectants [16], has been used also for testing the antibacterial activity of endodontic sealers [17-19].

Current sealers and materials for root-end filling are of highly varying chemical composition and antimicrobial properties. EndoRez is a methacrylate sealer with a potential to penetrate dentinal tubules. Reports vary regarding its antibacterial effects [12,20,21]. GuttaFlow is a flowable, thixotropic sealer which contains microscopic gutta-percha particles in a silicone base. Its antibacterial properties seem negligible [11,22,23]. Epiphany is a dual-cure resin sealer with a self-etching primer used with Resilon, a synthetic polymer-based core filling material. Epiphany-Resilon may have some antimicrobial activity [21]. AH Plus is an epoxy resin sealer. Its relatively high antimicrobial activity has been previously documented both by using the dentin block model and the direct contact test [10,19].

IRM was for many years the gold standard of root-end filling material. Mineral Trioxide Aggregate (MTA) is now a preferred root-end filling material because of its physical, chemical and biological properties [24,25]. *In vivo* studies have shown no statistically significant difference in the healing capacity between MTA and IRM [25,26]. MTA, as well as IRM, have antimicrobial activities against *Enterococcus faecalis* and *Candida albicans* [27,28]. The effectiveness of MTA and IRM against microbes in dentin has not been previously studied.

The objective of this study was to investigate *in vitro* the ability of the root-end filling materials MTA and IRM, different endodontic sealers and $Ca(OH)_2$ to kill *E. faecalis* in experimentally infected dentinal tubules.

Materials and methods

Test organism

The micro-organism used in this study was *Enterococcus faecalis* strain A197A, isolated from a tooth with persistent apical periodontitis [29]. Resistance to streptomycin was induced by culturing *E. faecalis* on agar plates with increased streptomycin concentration. The strain was stored in glycerine milk at $-70^{\circ}C$. For experiments, it was grown overnight on tryptic soy agar plates (TSA; Difco, Detroit, MI) with 2 mg/mL streptomycin (Sigma, St Louis, MO) and checked for purity by Gram-stain and growth on bile-esculin medium.

Dentin blocks

Ninety-four extracted single-rooted human teeth were used in this study. The teeth were stored in 0.01% NaOCl until use. The root surfaces were cleaned with a curette. Dentin blocks of 7 mm length were prepared by cutting off the crown and the root tip using a rotating diamond saw under water cooling. The crown and the root tip were discarded. Each root canal was enlarged with a size 2 (ISO size 90; Ø: 900 μm) Largo® Peeso Reamer (Dentsply-Maillefer, Ballaigues, Switzerland) under irrigation with water. In order to remove the smear layer, the dentin blocks were treated in an ultrasonic bath in 17% EDTA at pH 7.8 followed by 5% NaOCl, for 4 min each. The blocks were then sterilized by autoclaving for 20 min at $121^{\circ}C$ in distilled water. The specimens were dried and coated on their outer side with two layers of nail varnish. This procedure was performed in a sterile cabin. Sterility was checked by incubating the specimens in tryptic soy broth (TSB; Difco) for 24 h at $37^{\circ}C$.

Infection of dentin blocks

A stock suspension was prepared by incubating the streptomycin resistant strain of *E. faecalis* A197A in 50 mL TSB with 2 mg/mL streptomycin for 24 h at $37^{\circ}C$ in air. After 24 h of incubation, the suspension was kept at $4^{\circ}C$ (a temperature at which the broth does not freeze and the microbial activity remains relatively low) to be used as the stock suspension.

For use in the experiments, a 24 h-old suspension was prepared by inoculating 1 mL of the stock suspension into 50 mL TSB with 2 mg/mL streptomycin and incubating for 24 h at $37^{\circ}C$ in air. The dentin blocks were incubated in this suspension. The bacterial suspension was changed every second day for a period of 3 weeks. The purity of the cultures was checked once a week.

Procedure for testing

Ninety-four dentin blocks were randomly divided into eight groups (n = 12 for experimental groups and n = 10 for control). The excess fluid on the outer surface of the root segments was absorbed on a sterile gauze and the root canals were dried with sterile paper points. The tested materials are listed in Table I.

Mixing was according to the manufacturers' instructions. MTA and IRM were applied into the canal using a spatula and a small plugger. For group 3, pure $Ca(OH)_2$ powder was mixed with sterile distilled

Table I. Materials included in the study.

Group	Material	Code	Manufacturer
1	ProRoot MTA White	MTA	Tulsa Dental Products, Tulsa, OK
2	IRM	IRM	Dentslpy De Trey GmbH, Konstanz, Germany
3	Calcium hydroxide	$Ca(OH)_2$	Merck, Darmstadt, Germany
4	AH Plus + Gutta-percha	AH+	Dentslpy De Trey GmbH, Konstanz, Germany
5	EndoRez + Gutta-percha	ER	Ultradent Products Inc., South Jordan, UT
6	GuttaFlow + Gutta-percha	GF	Coltène/Whaledent AG, Altstätten Switzerland
7	Epiphany + Resilon	EpRe	Pentron Clinical Technologies, Wallingford, CT

water and applied into the root canal using a spatula and paper points. For groups 4–6, a gutta-percha cone size 80 and, for group 7, a Resilon cone size 70 was coated with a fresh mix of the corresponding sealer and introduced into the root canal. Excess was removed using a warm excavator. Infected dentin blocks left empty (group 8; nothing placed in the root canal) served as controls.

All specimens were incubated in air at 37°C in humid conditions. One half ($n = 6$) of the specimens in each experimental group were incubated for 1 day and the other half for 7 days. The controls were treated similarly; but the number of specimens was four for 1 day and six for 7 days. On completion of the incubation, all root canals were re-established using a sterile Largo® Peeso Reamer size 2, with an effort to avoid removing the root canal dentin. A sterile Largo® Peeso Reamer size 5 (ISO size 150; Ø: 1500 μm) attached to a slow-speed handpiece was used to enlarge the canal and the dentin powder was collected on sterile aluminum foil. Thus, a dentinal depth of 300 μm at each side of the root canal was sampled [(1500–900)/2 = 300 μm]. All sampling procedures were done under sterile conditions.

Microbiological analysis

The dentin powder samples were transferred to test tubes containing 2 mL of phosphate-buffered saline (PBS, Sigma) and each test tube was vortexed for 10 s. For assessment of the number of colony forming units (CFU) after incubation, 100 μL samples were taken and serial 10-fold dilutions made in 0.5% peptone water. From each dilution, droplets of 25 μL were cultured on TSA plates with 2 mg/mL streptomycin and incubated at 37°C in air for 48 h. Additionally, 25 μL samples from each test tube where the dentin chips were first collected were incubated on TSA plates with 2 mg/mL streptomycin at 37°C for 48 h. After 48 h of incubation, the plates were inspected for growth under a stereo-microscope (10× magnification) and bacterial colonies were counted. The counts were calculated as total CFUs per 2 mL sample. The detection limit of this experiment was 20 bacteria.

Finally, 10 mL brain heart infusion broth (BHI; Difco) was added to the primary test tubes containing the dentin powder/chips in PBS and incubated at 37°C in air for up to 1 week and checked daily for growth. The aim for doing this was to check whether viable bacteria below the detection limit existed. No quantitative analyses were performed from these cultures; only the presence or absence of growth was registered.

Data analysis

The CFU values were transformed to their corresponding log10 values. Statistical analysis was performed using Kruskal Wallis and Mann Whitney U-tests. Significance was set at the 5% level.

Results

Kruskal Wallis analysis suggested that significant differences were present among both day 1 and day 7 comparisons ($p = 0.000$ each). Cultivable bacterial numbers after treatment have been shown in Figure 1.

Day 1 comparison between the tested materials

Compared to the control, all materials significantly reduced the viable number of the bacteria ($p < 0.05$; Mann Whitney U; Table II). None of the samples that belonged to the groups AH+, IRM and EpRe exhibited bacterial growth on the agar plates. The groups ER and GF were similar to each other ($p > 0.05$); but yielded a significantly greater number of cultivable bacteria in comparison to the rest of the materials ($p < 0.05$). No significant difference was found between the groups $Ca(OH)_2$, AH+, IRM, EpRe and MTA ($p > 0.05$).

Day 7 comparison between the tested materials

Bacterial growth was seen in all groups. Compared to the control, all materials, except ER and GF, significantly reduced the viable number of bacteria ($p < 0.05$; Mann Whitney U; Table II). The groups ER and GF were similar to each other ($p > 0.05$) and,

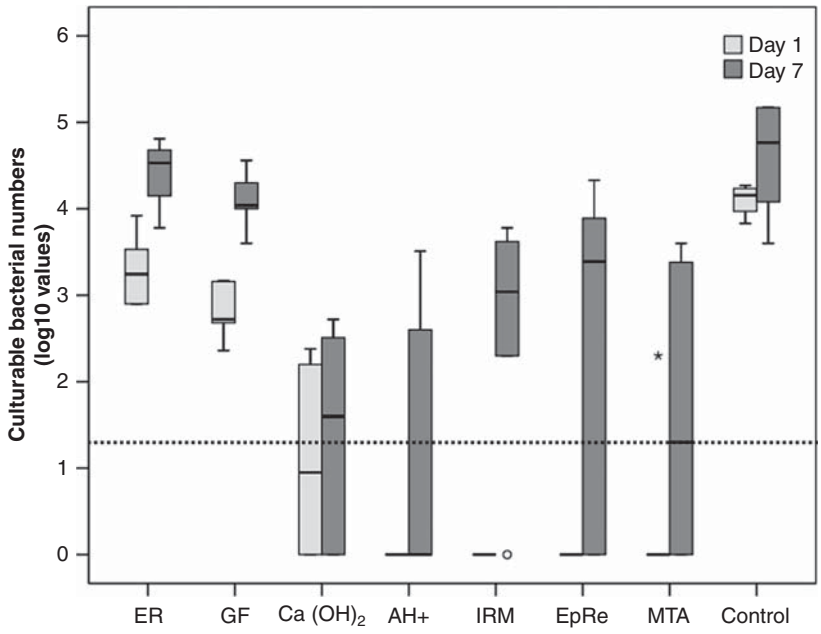

Figure 1. Box plot graph including sample minimum, lower quartile, median, upper quartile, sample maximum and outlier (the asterisk and the zero mark) for the number of CFU/2 mL (as log10 values) in each group at either day 1 or day 7 ($n = 6$, except day 1 control: $n = 4$). The bacterial detection limit has been shown with the dashed line (20 CFU/2 mL; log10 equivalent of 1.3).

again, yielded a significantly greater number of cultivable bacteria in comparison to the rest of the materials ($p < 0.05$; except no significant difference existed between GF and EpRe, $p > 0.05$). Again, no significant difference was found between the groups $Ca(OH)_2$, AH+, IRM, EpRe and MTA ($p > 0.05$).

Comparison between the day 1 and day 7 antibacterial activity of each material

Significant differences were found for each of the groups ER, GF, IRM and EpRe when their antibacterial activity at day 1 and day 7 were compared ($p < 0.05$; Mann Whitney U). The p-values were as follows: ER: 0.006, GF: 0.004, $Ca(OH)_2$: 0.618, AH+: 0.140, IRM: 0.007, EpRe: 0.022, MTA: 0.153 and control: 0.285.

Viable bacteria below detection limit

For all groups, primary tubes that have been added fresh broth for checking the presence of viable bacteria below the detection limit showed turbidity, suggesting that the specimens, although yielding non-cultivable

Table II. p-values for group comparisons for each day 1 (upper row) and day 7 (lower row).

	ER	GF	$Ca(OH)_2$	AH+	IRM	EpRE	MTA
GF	0.054						
	0.109						
$Ca(OH)_2$	*0.004*	*0.006*					
	0.004	*0.004*					
AH+	*0.002*	*0.002*	0.059				
	0.003	*0.003*	0.607				
IRM	*0.002*	*0.002*	0.059	1.000			
	0.005	*0.010*	0.076	0.097			
EpRE	*0.002*	*0.002*	0.059	1.000	1.000		
	0.016	0.054	0.191	0.171	0.686		
MTA	*0.003*	*0.003*	0.295	0.317	0.317	0.317	
	0.004	*0.005*	0.739	0.531	0.289	0.319	
Control	*0.019*	*0.010*	*0.010*	*0.004*	*0.004*	*0.004*	*0.006*
	0.423	0.107	*0.004*	*0.003*	*0.010*	*0.016*	*0.005*

Statistical significance was set at 5% (Mann Whitney U-test) and significant values have been marked with italics.

bacteria on agar plates, actually contained viable bacteria.

Discussion

Antimicrobial activity of root filling materials may be advantageous when the pulp canal system is infected. In this study, the antimicrobial activity of various root canal sealers, root-end filling materials and a root canal medicament has been examined; all materials exerted varying degrees of antibacterial activity which generally tended to decrease with time.

The dentin block model has been designed to assess the antimicrobial activity of solutions or substances diffusing into dentinal tubules. In the study by Heling and Chandler [18], the dentin block model was used to test the antibacterial activity of root canal sealers. The most prominent antimicrobial activity was demonstrated by AH-26 (the forerunner of AH Plus) after 1 day as well as 7 days of treatment, with no statistically significant difference between its effects at the two time intervals. However, more bacteria were recovered after 7 days than after 1 day with AH-26, similar to what has been found also in the present study. In the study by Saleh et al. [19], AH Plus was found to eliminate cells of *E. faecalis* from infected dentin blocks to a distance of 300 μm after 7 days. The antibacterial effect of AH Plus and Epiphany sealer was shown in the present study and the findings of this study support those of previous studies that have tested AH Plus and Epiphany [10,11,19,21]. The antibacterial component of these two sealers is not known, but it can be speculated that the resin monomers may be involved.

Blocks filled with AH Plus yielded, without statistical significance, fewer bacteria compared with the $Ca(OH)_2$ group after 7 days. It may, therefore, be speculated that the antimicrobial effect of this sealer can compensate for the inter-appointment medication, thus favoring a one-step treatment approach. However, $Ca(OH)_2$ also has other mechanisms of effect such as dissolution of necrotic or vital residual tissue in the root canal system and neutralization of the biological activity of microbial antigens [30].

Another interesting finding was that the sealers EndoRez and GuttaFlow were similar to each other, less effective than the other materials and similar to the control at day 7. These findings, suggesting a moderate initial antibacterial activity for these sealers, are partly in line with findings of previous studies that have attributed no to mild or moderate antibacterial activity to these sealers [11,12,20,22,23]. Our findings for EndoRez differ from those who have found strong antibacterial activity, even after a setting period of 7 days [21].

In the present study, one focus of interest was on the root-end filling materials IRM and MTA. IRM contains eugenol, which has antimicrobial activity. Maltezos et al. [31] showed that Super-EBA, which somewhat resembles IRM in chemical composition and properties, leaked significantly more in a bacterial leakage experiment than MTA and Epiphany-Resilon and speculated on the potential use of Epiphany-Resilon as a root-end filling material. In the present study, Epiphany-Resilon demonstrated similar antibacterial activity to IRM and again no significant difference was found between these and MTA. The antibacterial effect of MTA is supposed to be due to its high pH. In contrast to the inhibition by dentin powder of most of the conventional disinfectants, it was shown that the presence of dentin powder enhances the antibacterial activity of MTA, a finding supporting its clinical use from an antimicrobial standpoint [32].

The most effective materials tested in this study (i.e. calcium hydroxide, AH Plus and MTA) caused a 100- to more than 1000-fold reduction in bacterial counts compared to the control group. Although not completely bacteria-free, the reduction may be biologically important, particularly if it is accompanied by a tight seal. It is also worth noting that, although $Ca(OH)_2$ has repeatedly been shown to fail in eradicating all microbes from the infected dentin, the present study, in line with the findings of Saleh et al. [19], show that the reduction of CFU values at day 7 was greater than with most of the other root filling materials, except AH Plus and MTA. The antimicrobial performance of $Ca(OH)_2$ and MTA was similar at both 1-day and 7-day periods. This is probably because of the similar antibacterial mechanisms of the materials, as $Ca(OH)_2$ is formed when MTA is mixed with water; thus, both act as being strongly alkaline materials [33].

The amount of recovered bacteria increased from 1-day to 7-days of treatment in all experimental groups; the increase was not significant in the $Ca(OH)_2$, AH Plus and MTA groups. Previous studies have also concerned the antibacterial activity of sealers at short-term and at relatively longer periods; and found that variations between sealers may be seen according to the sealer type; but the general idea is that, in most instances, the antibacterial activity is greater at the freshly-mixed-state, but reduces with time [34,35]. The reason or mechanisms by which viable bacteria re-populate apparently disinfected areas in the dentin is not clear. It may be speculated that the initial toxicity of the tested materials during setting inhibits the growth of bacteria recovered from the inner zones of the dentin. Possibly, the bacteria become shocked by the toxic components leaching out from the fresh material (during 1-day treatment) and cannot grow when plated on the agar medium. However, as the material sets (during 7-days of treatment), the antibacterial activity of the material reduces, the once-shocked-bacteria gradually adapt to the environment and regain their ability to grow when plated on the agar. Another explanation for the

increase in CFUs may be the regrowth of viable bacteria from areas outside the primary killing zone towards the main canal after the setting is complete. As *E. faecalis* has the ability to derive the nutrients necessary for growth from the organic components of the dentin [36], this possibility cannot be excluded.

The lumen of the canal was enlarged to a size 90 before infecting with the bacteria, a size relatively sufficient to eliminate irregularities, obtain a round canal and manipulate sealers/fillings/medicaments uniformly into the canal. In this condition, the root-end filling materials, MTA and IRM, and the root canal medicament, $Ca(OH)_2$, were placed in relatively larger amounts and the sealers, AH Plus, EndoRez, GuttaFlow and Epiphany, in smaller amounts since they were inserted with a core material. From an antimicrobial standpoint, the amount of the material placed in the root canal may be important, especially before the setting reaction is complete. In the fresh state, leaching of more active ions/compounds is expected than when the material set. Thus, a material placed in greater quantities into the canal is likely to act as a depot and provide more antimicrobially active component. Another point, as mentioned earlier in the text, is the buffering by dentin of the antimicrobials [13-15]. It can also be expected that a material placed into the canal as depot will be less affected by this buffering effect compared to one placed as thin film. In the context of this discussion, the materials placed in depot in this study [MTA, IRM and $Ca(OH)_2$] were found to exhibit relatively strong initial antimicrobial activities. The sealers AH Plus and Epiphany, although thinner, had definitely strong initial antimicrobial activities.

The strain tested in this study (strain A197A) was a clinical isolate from a root canal with persistent infection [29]. This strain has been often used as the test strain in previous studies as well [10,13-15,19,22]. However, it should be kept in mind that results from one study that has tested one particular strain cannot be extrapolated; phenotypic differences between strains are possible. This study has only compared the materials in the defined set-up.

In conclusion, all materials tested in this study initially (day 1) exerted definite antibacterial activity which tended to decrease with time. Later (day 7), the antimicrobial activity was either negligible (ER and GF), decreased but still significant (IRM and EpRe) or remained similar to the initial (calcium hydroxide, AH+ and MTA). Regrowth of bacteria in filled root canals, as seen in this study, suggests the use of filling materials with sustained antibacterial activity be preferred.

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