Survival of *Enterococcus faecalis* in infected dentinal tubules after root canal filling with different root canal sealers *in vitro*

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Abstract

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Aim To investigate the ability of different endodontic sealers and calcium hydroxide to kill bacteria in experimentally infected dentinal tubules.

Methodology Fifty-six human root segments were enlarged to size 2 (ISO size 090) Largo® Peeso Reamer. After treatment with 17% EDTA and 5% NaOCl for 4 min each, the specimens were infected with *Enterococcus faecalis* for 3 weeks. The roots were divided into eight groups and filled with gutta-percha and AH Plus (AH); Grossman's sealer (GS); Ketac-Endo (KE); Apexit (AP); RoekoSeal Automix (RSA); or RoekoSeal Automix with an experimental primer (RP), or calcium hydroxide (CH) only. One group of specimens was left unfilled for control (CT). Following storage in humid conditions at 37 °C for 7 days, the root canals were re-established with new sterile Largo® size 2. Dentine samples from each canal were then collected using a sterile size 5 (ISO size 150) Largo® Peeso Reamer. The number of colony-forming units (CFU) was determined for each sample.

Results The mean $\log_{10}$ CFU in all test groups was significantly lower ($P < 0.05$) than that in the CT group. Root filling with AH and GS killed bacteria (mean CFU = 0) in the dentinal tubules. The mean $\log_{10}$ CFU for the CH group (0.53) was lower than that of RSA, AP, RP and KE (1.36, 1.40, 1.46 and 1.94, respectively), but only the difference between the CH and the KE groups was statistically significant ($P < 0.05$).

Conclusion Root fillings *in vitro* with gutta-percha and AH or GS were effective in killing *E. faecalis* in dentinal tubules. Other endodontic sealers, as well as CH, were less effective.

Keywords: calcium hydroxide, *E. faecalis*, root canal sealers, root dentine infection.

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Introduction

Bacteria are the main causative factor in the development of periapical inflammation (Kakehashi *et al.* 1965, Sundqvist 1976). The main objective of endodontic therapy is, therefore, to eliminate bacteria from the infected root canal and to prevent reinfection. Complete chemomechanical preparation remains the most important step in root canal disinfection. Careful obturation is essential to avoid reinfection of the root canal space.

Even after thorough cleaning, shaping and irrigation with disinfectants, total elimination of bacteria is difficult to achieve in all cases (Byström & Sundqvist 1983; 1985). These procedures undoubtedly reduce the number of viable microorganisms in the root canal, but the complex anatomy of the root canal system often makes complete debridement impossible.

It is generally believed that remaining bacteria can be eliminated or be prevented from repopulating the root canal space by placing an interappointment dressing such as calcium hydroxide (CH) in the prepared canal (Byström *et al.* 1985, Chong & Pitt Ford 1992). It has,

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however, been demonstrated that CH does not consistently produce bacteria-free root canals and even may allow regrowth in some cases (Reit & Dahlén 1988, Ørstavik & Haapasalo 1990, Ørstavik *et al.* 1991, Peters *et al.* 2002). The physicochemical properties of CH may limit its effectiveness in disinfecting the entire root canal system. In addition, it is not effective against all bacterial species found in root canal infections (Siqueira & Lopes 1999).

Remaining microorganisms may also be eliminated or rendered harmless by entombing them through complete obturation with gutta-percha points and sealer after chemomechanical cleaning and disinfection (Sundqvist & Figdor 1998). The remaining microorganisms may be killed by the antimicrobial activity of the sealer or the Zn^{2+} ions of gutta-percha points (Moorer & Genet 1982, Heling & Chandler 1996, Kaplan *et al.* 1999, Siqueira *et al.* 2000), or they may be deprived of nutrition and space to multiply (Sundqvist & Figdor 1998).

The antibacterial effect of various endodontic sealers has been previously studied on agar plates, and all tested sealers have shown some antibacterial effect (Al Khatib *et al.* 1990, Siqueira *et al.* 2000). In addition to providing physical obturation, the root canal filling may therefore supplement the chemomechanical preparation in disinfection of the root canal space. The antibacterial properties of sealers may also be advantageous in clinical situations of persistent or recurrent infection (Ørstavik 1988).

The objective of this study was to investigate *in vitro* the ability of root fillings with gutta-percha and different endodontic sealers, and CH, to kill bacteria in experimentally infected dentinal tubules.

Materials and methods

Root dentine specimens

Extracted single-rooted human teeth were used in the study. The teeth were stored in 0.01% NaOCl at 4 °C after cleaning the root surface with curettes. Root segments with a length of 7 mm (Fig. 1) were prepared by cutting off the root tip and the crown 2–3 mm below the cemento–enamel junction, using a rotating diamond saw (Accutom, Struers, Copenhagen, Denmark) under water cooling. Each root canal was enlarged to size 2 (ISO size 090) Largo® Peeso Reamer (Dentsply Maillefer, Ballaigues, Switzerland) under irrigation with distilled water, resulting in the preparation of an apical box. Organic and inorganic debris including the smear layer were removed by treatment in an ultrasonic bath (Finn-Sonic m03/m, Lahti, Finland) in 17% EDTA followed by 5% NaOCl, for 4 min each (Fig. 2). The test specimens were sterilized by autoclaving for 20 min at 121 °C. The specimens were then blotted dry and coated on their outer, periodontal surface with nail varnish under sterile conditions. Sterility was checked by incubating each specimen in 2 mL of sterile tryptone soy broth (TSB; Oxoid Ltd, Basingstoke, UK) with 2 mg mL^{-1} streptomycin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) for 24 h at 37 °C.

Figure 1 Dentine specimen with root filling in place.

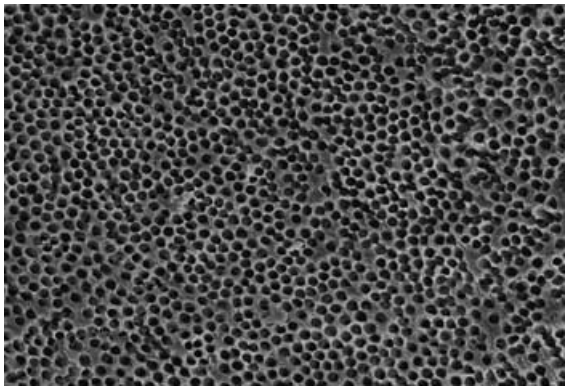

Figure 2 A scanning electron micrograph showing open dentinal tubules after treatment with NaOCl and EDTA (magnification × 500).

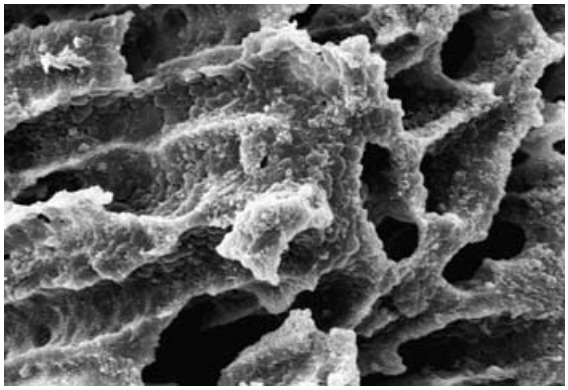

Figure 3 A scanning electron micrograph showing *E. faecalis* cells in dentinal tubules after a 3-week infection period (magnification × 5000).

Infection of root specimens

A strain of *E. faecalis*, A197A, adapted to and maintained on TSB with 2 mg mL^{-1} streptomycin, was used as a test organism. The test specimens were transferred into 2 mL of TSB with 2 mg mL^{-1} streptomycin inoculated with 200 μL of a 24-h-old *E. faecalis* suspension. Under strict asepsis, the bacterial suspension was changed every second day for a period of 3 weeks. The purity of the cultures was checked regularly. For visual control (CT) of infection, selected specimens were fractured and processed for analyses in the scanning electron microscope (Philips XL 30, Eindhoven, the Netherlands) (Fig. 3); other specimens were processed for light microscopic studies after Brown & Brenn staining for bacteria (Haapasalo & Ørstavik 1987).

Procedure for testing

Fifty-six root dentine specimens were randomly divided into eight groups ($n = 7$). The root canals were blotted dry with sterile paper points. Five sealers of different chemical composition were tested (groups 1–6, Table 1). The sealer to be tested was mixed according to the manufacturer's instructions. A gutta-percha cone size 90 coated with the freshly mixed sealer was introduced into the prepared canal with a tweezer until fully seated. For group 7, pure CH powder was mixed with distilled water in a powder-to-liquid ratio of 3 : 2 (v/v) and then condensed into the root canal with sterile paper points. Infected specimens with no medicament served as controls (group 8). All specimens were incubated at 37 °C for 7 days under humid conditions.

On completion of incubation, all root canals were re-established with sterile Largo® Peeso Reamers size 2. A new sterile Largo® Peeso Reamer size 5 (ISO size 150) was then used to remove dentine powder (300 μm into the dentine) from each canal with the specimen held in sterile gauze.

Microbiological analysis

The dentine powder obtained from each specimen was immediately collected in sterile Petri dishes. The samples were then transferred to test tubes containing 2 mL of phosphate buffered saline (PBS; Bio Whittaker, Verviers, Belgium), vortexed for 10 s and diluted to a concentration of 10^{-4}. Portions of 25 μL were inoculated onto TSB agar with 2 mg mL^{-1} streptomycin. Following incubation for 48 h at 37 °C, visible colonies were counted and the total colony-forming units (CFU) calculated. When growth occurred, the bacteria were subcultured on TSB agar plates with 2 mg mL^{-1} streptomycin and checked for purity and identity of *E. faecalis*.

Data analysis

The CFU values were transformed to their $\log_{10}$ values. The mean values of $\log_{10}$ CFU with the standard deviation (SD) were calculated. Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test for multiple comparisons. Significance was set at the 5% level.

Results

The mean $\log_{10}$ CFU for the various test groups is illustrated in Fig. 4. Bacteria were found in all CT samples.

Table 1 Materials used

Sealer	Code	Chemical composition	Manufacturer	Batch number
Grossman's sealer	GS	Zinc oxide-eugenol-based	By prescription (NIOM laboratory, Haslum, Norway)	2
Apexit	AP	Calcium hydroxide-based	Vivadent, Schaan, Liechtenstein	912697
Ketac-Endo	KE	Glass ionomer-based	Espe, Seefeld, Germany	FW0055094
AH Plus	AH	Resin-based	Dentsply DeTrey GmbH, Konstanz, Germany	9810000713
RoekoSeal Automix	RSA	Silicone-based	Roeko, Langenau, Germany	B239b/B240b
RoekoSeal Automix + Primer	RP	Silicone-based	Roeko, Langenau, Germany	M496

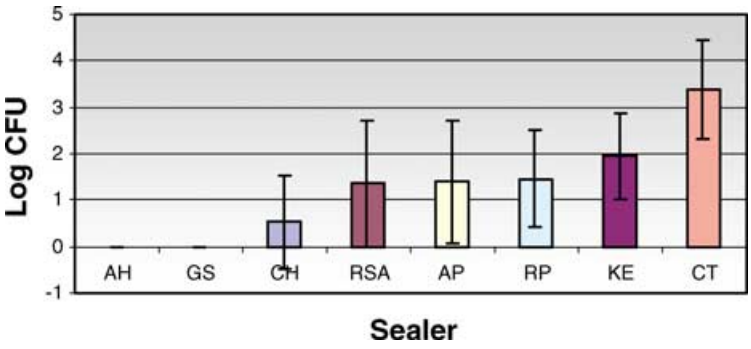

Figure 4 Mean $\log_{10}$ of the number of CFU in root dentine collected from test and CT specimens. (AH, AH Plus; GS, Grossman's sealer; CH, calcium hydroxide; RSA, RoekoSeal Automix; AP, Apexit; RP, RoekoSeal Automix + Primer; KE, Ketac-Endo.)

The mean $\log_{10}$ CFU in all test groups was significantly lower than that in the CT group ($P < 0.05$). AH Plus (AH) and Grossman's sealer (GS) totally killed bacteria (mean CFU = 0) in the dentinal tubules after application for 7 days. The mean $\log_{10}$ CFU for the CH group (0.53) was lower than that of the RoekoSeal Automix (RSA), Apexit (AP), RoekoSeal Automix with the experimental primer (RP) and Ketac-Endo (KE) (1.36, 1.40, 1.46 and 1.94, respectively), but only the difference between the CH and the KE groups was statistically significant ($P < 0.05$). The mean $\log_{10}$ CFU for the KE group was significantly higher than that for the AH and GS groups ($P < 0.05$).

Discussion

Enterococcus faecalis was chosen as the test organism because it is associated with persistent apical inflammation in clinical situations (Molander *et al.* 1998, Sundqvist *et al.* 1998). The dentine block model by Haapasalo & Ørstavik (1987), with some modifications, was used. *E. faecalis* has been found suitable for experimental penetration into dentinal tubules, and 3 weeks of incubation with *E. faecalis* has been shown to produce a dense infection of the dentinal tubules, easily reaching 300–400 μm (Haapasalo & Ørstavik 1987). Prolonged infection mainly leads to more tubules being infected, whereas the average depth of penetration of the tubules by bacteria has been found to increase only slowly with time (Haapasalo & Ørstavik 1987).

Eliminating bacteria from the root canal system is essential for long-term treatment success. Previous studies have indicated that instrumentation and irrigation with NaOCl cannot produce bacteria-free root canals (Byström & Sundqvist 1985). When, however, followed by application of an antimicrobial dressing, like CH, applied for a suitable length of time before root filling, bacteria can be reliably eliminated from the root canal (Byström *et al.* 1985). Intracanal medication with CH for 4 weeks (Byström *et al.* 1985), or 1 week (Sjögren *et al.* 1991), effectively eliminated bacteria in the root canal in up to 100% of the cases. On the other hand, it has been argued that even in cases that yield negative cultures clinical failure still may occur (Sjögren *et al.* 1997). A possible explanation is that bacteria located in isthmuses, dentinal tubules and ramifications may have been inaccessible to sampling (Siqueira & Lopes 1999).

Recently, doubt has been raised about the necessity of an antimicrobial intracanal dressing (Peters *et al.* 1995, Weiger *et al.* 2000, Peters & Wesselink 2002, Peters *et al.* 2002). Several studies (Byström *et al.* 1985, Haapasalo & Ørstavik 1987, Safavi *et al.* 1990, Heling *et al.* 1992) have shown the limited capability of CH to completely kill bacterial cells inside dentinal tubules. The present study confirms this finding. Application of CH for 7 days reduced but did not effectively kill *E. faecalis* in infected dentinal tubules.

The antimicrobial effects of CH may relate directly to its high pH (Byström *et al.* 1985). To be effective against bacteria inside dentinal tubules, hydroxyl ions from CH must diffuse into dentine and reach sufficient pH levels to be lethal to bacteria. The low solubility and diffusibility of CH, as well as the dentine buffering ability, may make it difficult to reach an increase in the pH capable of eliminating bacteria located within dentinal tubules or enclosed in anatomical variations (Siqueira & Lopes 1999).

The root filling serves to prevent infection by acting as a barrier to further microbial challenges, to entomb any surviving bacteria in the root canal system, and to stop periapical tissue fluids from reaching bacterial cells in the root canal and maintaining their survival (Sundqvist & Figdor 1998). Moreover, some endodontic sealers elicit an antibacterial activity *in vitro* (Heling & Chandler 1996, Kaplan *et al.* 1999) that may contribute to the destruction of intracanal microorganisms.

The results of the present study showed that the use of GS and AH in root fillings *in vitro* killed all bacteria in the dentinal tubules within the zone of 300 μm around

the root canal. The results confirm and expand previous findings by Ørstavik (1988) and Heling & Chandler (1996). Sealers containing zinc oxide–eugenol or epoxy/amines have previously been shown to be the most effective against microorganisms (Heling & Chandler 1996, Kaplan *et al*. 1999, Leonardo *et al*. 1999).

Root fillings with the other endodontic sealers tested (KE, AP, RSA and RP) were less effective than those with CH in killing bacteria. The antimicrobial activity of AP may be based on its content of CH, but the pH rise by AP may not be sufficient to kill *E. faecalis*. The antimicrobial activity of KE is assumed to rely on fluoride-ion release, but the quantities of fluoride released may not be sufficient to reach a concentration that effectively kills bacteria. A weak antimicrobial activity of KE has been reported by Heling & Chandler (1996). The antimicrobial effect of RSA has not been previously tested. The present study indicates a limited ability of RSA to kill *E. faecalis*.

Ideally, the root canal sealer should have both antibacterial activity and low toxicity to the surrounding tissues and cells. Root canal sealers with strong antibacterial activity have been found to be cytotoxic and even mutagenic (Geurtsen & Leyhausen 1997). The antibacterial components of the sealer do not have selective toxicity against microorganisms; they will also exert toxic effects on host cells. The sealers demonstrating a strong antibacterial activity in the present study (AH and GS) have previously been shown to possess relatively low cytotoxicity (Schwarze *et al*. 2002, Camp & About 2003).

Differences in healing potential for teeth that are treated in one or two visits, the latter with placement of an intracanal disinfectant, appear to be small (Trope *et al*. 1999, Weiger *et al*. 2000, Peters & Wesselink 2002). The results of the present study indicate that obturation with gutta-percha and sealers that are capable of eliminating residual bacteria in the root canal system may improve the treatment results in both approaches.

Conclusions

The use of AH and GS in root fillings *in vitro* was effective in killing *E. faecalis* in experimentally infected dentinal tubules within the zone of 300 μm around the root canal. Other endodontic sealers, as well as CH, reduced the numbers, but did not effectively kill bacteria in infected dentinal tubules.

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