In vitro antimicrobial effect of chlorhexidine-impregnated gutta percha points on *Enterococcus faecalis*

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Abstract

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Aim To evaluate the *in vitro* antimicrobial effect of chlorhexidine-impregnated gutta percha points, Roeko activ point (Roeko, Langenau, Germany) on *Enterococcus faecalis*.

Methodology Human maxillary premolar roots were prepared with .04 rotary ProFile instruments to a master apical file size 40, autoclave-sterilized and then infected with *E. faecalis* (ATCC 29212) for 3 weeks. Baseline controls were carried out verifying negligible effects of plain gutta percha cones on *E. faecalis*. Subsequent to intracanal placement of calcium hydroxide, 'activ points' or saline (positive control) and the 2-week incubation in 54 root specimens, dentine sampling at depths of 100 and 250 μm was carried out using .04 rotary ProFile instruments at sizes 60 and 90 to assess the quantity of bacteria present. Inactivating agents were used prior to sampling and the colony-forming units (CFU) of *E. faecalis* were then plate-counted after culturing. Statistical analysis was completed using the paired *t*-test.

Results In comparison to the positive control, treatment with calcium hydroxide ($P = 0.000$ and 0.000) or activ points ($P = 0.000$ and 0.002) produced significantly lower colony counts of *E. faecalis* at dentine depths of 100 and 250 μm, respectively. Calcium hydroxide (2.10×10^2 CFU mL^{-1}) was significantly more effective than activ points (1.58×10^3 CFU mL^{-1}) at 100 μm ($P = 0.013$), but not at 250 μm ($P = 0.353$). Neither of these two medications was able to eliminate *E. faecalis* completely.

Conclusions Chlorhexidine-impregnated activ points did not possess an *in vitro* inhibitory activity strong enough to eliminate *E. faecalis* completely from infected dentinal tubules.

Keywords: activ point, antimicrobial effect, *E. faecalis*, intracanal medicament.

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Introduction

Bacteria and their by-products are considered the primary aetiologic agents of necrotic pulps and apical periodontitis (Kakehashi *et al.* 1965, Sundqvist 1976). Accordingly, the aim of root-canal treatment is the elimination of infection from the canal system. Similarly, the main cause of root-canal treatment failure is the persistence of microorganisms after therapy (Molander *et al.* 1998, Sundqvist *et al.* 1998) or the reinfection of the root-canal system because of inadequate coronal seal (Ray & Trope 1995).

Infections of untreated root canals with necrotic pulps and apical periodontitis are typically polymicrobial, with approximately equal proportions of Gram-positive and Gram-negative anaerobic bacteria (Sundqvist 1976). The microbial flora of the retreatment cases, however, has been characterized as monoinfections of predominantly Gram-positive microorganisms, with approximately equal proportions of facultative and obligate anaerobes (Molander *et al.* 1998, Hancock

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et al. 2001). *Enterococcus faecalis*, a facultative Gram-positive coccus, is the most frequently isolated species and may also sometimes be the only isolate (Molander *et al.* 1998, Sundqvist *et al.* 1998, Peciuliene *et al.* 2000, Hancock *et al.* 2001).

The use of intracanal calcium hydroxide as an adjunct to chemo-mechanical instrumentation (Bystrom & Sundqvist 1981, Bystrom & Sundqvist 1983), and prevention of culture reversal (Bystrom & Sundqvist 1985) in teeth with primary apical periodontitis has been well established. However, several studies have shown that *E. faecalis* is relatively resistant to calcium hydroxide (Bystrom *et al.* 1985, Haapasalo & Orstavik 1987), which may partially explain the lower prognosis of retreatment cases with secondary apical periodontitis (Sjogren *et al.* 1990).

Chlorhexidine, a cationic bisguanide with the ability to adsorb onto dentine (Parsons *et al.* 1980), is considered a broad-spectrum antimicrobial agent. It acts by adsorbing onto the microorganism cell wall and causing intracellular component leakage. Chlorhexidine has been suggested as an effective irrigant (Delany *et al.* 1982, Jeansonne & White 1994) and an intracanal medicament because of its ability to disinfect dentinal tubules against *E. faecalis* (Heling *et al.* 1992, Vahdaty *et al.* 1993).

In addition to the gel form of chlorhexidine suggested for use as an intracanal medication (Siqueira & Uzeda 1997), a new formulation of a chlorhexidine-impregnated gutta percha point, 'activ point' (Roeko, Langenau, Germany), has recently been marketed. According to the manufacturer, 'activ points' contain gutta percha matrix embedded with 5% chlorhexidine diacetate. This innovation allows ease of introduction and retrieval from the root canal. However, previous studies on 'activ points' only evaluated the effect of direct exposure to a suspension broth of bacteria (Podbielski *et al.* 2000, Petschelt *et al.* 2002). As there has been no study establishing the antimicrobial effect of 'activ points' against bacteria within the infected dentinal tubules, this study was undertaken to investigate, using an *in vitro* infected dentine model, the antimicrobial effect of 'activ points' against *E. faecalis* when used as an intracanal medicament.

Materials and methods

Teeth selection

Human permanent maxillary premolars extracted for orthodontic reasons with no existing caries or restorations, and the buccal and palatal roots clearly separated were collected and stored in physiological saline. These teeth were included based on the criteria of root length ranging between 11 and 13 mm measured from the lowest level of the cemento-enamel junction (CEJ) to the mature root apices and root curvature less than 15°, as well as uniform dentine thickness verified radiographically in the buccolingual and mesiodistal dimensions. Adherent soft tissue was mechanically removed without damaging the root surface. Inspection was further carried out to ensure that there were no cracks, fractures or areas of root resorption.

Teeth preparation

Decoronation and separation of the two roots was performed using a high-speed diamond bur. All roots were adjusted to 10 mm length and confirmed to have a minor apical foramen of size 20. The root canals were instrumented to 9 mm using rotary .04 ProFiles (Dentsply Maillefer, Ballaigues, Switzerland) in conjunction with 6 mL of 1% sodium hypochlorite irrigation (Ricochlor, Orion Laboratories, Welshpool, Western Australia, Australia). At the completion of canal preparation to a master apical file of .04 ProFile size 40, the teeth were treated in an ultrasonic bath with 17% EDTA (Pulpdent Corporation, Watertown, USA) for 4 min and 5.25% sodium hypochlorite for 4 min to remove the smear layer (Haapasalo & Orstavik 1987). Copious irrigation with distilled water was carried out to remove any remaining EDTA and sodium hypochlorite.

Sterility and asepsis control

Each root specimen was placed in an individual Bijou bottle containing 2 mL of Brain Heart Infusion (BHI) broth (Becton Dickonson, Franklin Lakes, USA) and autoclaved for 20 min at 121 °C. They were then incubated for 48 h at 37 °C and inspected daily to ensure that the BHI broth showed no signs of turbidity as a sterility control prior to proceeding with the subsequent stages of the experiment.

As a further step to assess the asepsis protocol used throughout the experimental procedures, five additional instrumented and sterilized roots in BHI broth were incubated for 3 weeks at 37 °C. The roots were washed with saline in a vortex mixer for 10 s and the root surface was disinfected with 70% ethanol and dried with sterile gauze. Five microlitres of sterile saline was placed in each root canal prior to 2-week incubation of the root specimen wrapped with saline-dampened sterile gauze within an Eppendorf tube at 37 °C under humid

Table 1 Statistical analysis of the number of CFU for the baseline control experimental groups

	Dentine depths						
	100 μm*		250 μm*			Mann–Whitney (dentine depths)	
Treatment ($n = 6$)	Mean ± SD	Median	Mean ± SD	Median	Pair-wise comparison	100 μm	250 μm
Activ points	1205 ± 1154	778	1027 ± 669	1282	Activ points and saline	0.004	0.004
Saline (positive control)	31650 ± 34377	21225	16083 ± 16992	10600	Saline and gutta percha	1.000	0.337
Gutta percha	21158 ± 4689	20225	14408 ± 4872	14175	Gutta percha and activ points	0.004	0.004

*Kruskal–Wallis test $P = 0.003$.

conditions. At the end of the incubation, bacterial sampling from the dentinal walls performed in accordance to the prescribed protocol in the experiment showed no bacterial growth.

Inoculation control of *E. faecalis*

A pure culture of the test strain, *E. faecalis* (ATCC 29212; Global Bioresource Center™, Manassas, Virginia, USA) was prepared in sterile BHI broth and adjusted using a spectrophotometer (Spectronic Genesys 5; Milton Roy, Ivyland, USA) to an optical density of 1.0 at 600 nm corresponding to 1.15×10^8 colony-forming units (CFU) mL^{-1}. The prepared root specimen in each Bijou bottle was incubated at 37 °C with the *E. faecalis* broth changed every 3 days for the total inoculation period of 3 weeks (Akpata & Blechman 1982, Haapasalo & Orstavik 1987). Random sampling and plating of the inoculum to confirm the viability and purity of the *E. faecalis* broth was done weekly using a bacterial identification kit (API 20 strep; bioMerieux, MO, USA). In addition, the root-canal dentine of another five infected roots was sampled according to the dentine sampling protocol after the 3-week inoculation period to validate the effectiveness of the dentine inoculation protocol. Mean counts of 1.33×10^5 and 6.85×10^4 CFU mL^{-1} were recovered at dentine depths of 100 and 250 μm, respectively. All procedures were carried out under strict aseptic conditions in a laminar airflow chamber (Gelman Sciences, Ann Arbor, Michigan, USA).

Baseline effect of gutta percha

In order to ascertain the extent of the speculated antibacterial effect of gutta percha cones (Moorer & Genet 1982), which form the inner core of the 'activ point' coated with chlorhexidine diacetate, a baseline control experiment was carried out. Three groups of six inoculated root samples each were incubated for 2 weeks with intracanal 'activ points', gutta percha cones and saline (positive control), and the effect on *E. faecalis* was compared (Table 1). Statistical analysis using Kruskal–Wallis and Mann–Whitney tests showed negligible effects ($P = 1.000$ and 0.337) of the 9-mm gutta percha cones size 40 (Top color, Roeko, Langenau, Germany) on *E. faecalis* (2.02×10^4 and 1.42×10^4 CFU mL^{-1}), and similar effects to that of the positive control (saline group; 2.12×10^4 and 1.06×10^4 CFU mL^{-1}).

Experimental proper and treatment groups

Twenty-seven teeth were selected for the experiment proper with each pair of roots randomly assigned to two of the three treatment groups. The three treatment groups, each of a sample size of 18 roots, were prepared according to the following protocols prior to the 2-week humid incubation at 37 °C.

- Group A (calcium hydroxide): 10 mg of calcium hydroxide powder (USP; Pulpdent Corporation, Watertown, USA) mixed with 10 μL of distilled water into a creamy paste was placed into the root canal of each root specimen with a lentulo spiral.
- Group B ('activ points'): An 'activ point' size 40 trimmed to a 9 mm length was inserted into the root canals according to the manufacturer's instructions (Roeko 1999) in the presence of 5 μL distilled water.
- Group C (positive control): 5 μL of sterile saline was syringed into the root canals of the specimens.

Preparation for bacterial sampling

The specimens were retrieved from the Eppendorf tubes, and intracanal materials were removed according to the following protocol prior to the dentine sampling.

- Group A (calcium hydroxide): Calcium hydroxide paste was removed from the canals by rotating a rotary ProFile .04 taper size 40 instrument for 20 s (Han *et al.* 2001). The canals were then flushed with 3 mL of 0.5% citric acid (Moller 1966) followed by 3 mL of sterile saline.
- Group B ('activ point'): The 'activ point' was removed using a pair of sterile tweezers. The canal was then flushed with 3 mL of 3% Tween 80 (Sigma Chemical,

St Louis, USA) and 0.3% L-α-lecithin (Sigma Chemical, St Louis, USA), and then with 3 mL of sterile saline (Zamany & Spangberg 2002).

- Group C (positive control): The canals were flushed with 6 mL of sterile saline.

Dentinal sampling and bacteriological evaluation

New sterile rotary ProFile .04 tapers sizes 60 and 90 were used at 300 r.p.m. to remove the dentine from the root-canal wall in the presence of the irrigated BHI broth. The dentine sample was collected within 5 mL of BHI broth in a test tube. The suspension was then vortexed for 10 s and serially diluted. Aliquots of 100 μL were subsequently plated onto Tryptic Soy Agar (TSA) blood agar plates (Biomedia Laboratories, Melaka, Malaysia) in duplicates and incubated at 37 °C for 24 h. The quantity of CFU grown was counted and the identity was evaluated using the API 20 strep (bio Merieux) bacterial identification kit.

Statistical analysis

The results obtained from groups A–C were subjected to logarithmic transformation to obtain a near-normal distribution (Figs 1–4). Thereafter, the paired *t*-test was used to investigate the differences in the CFU of *E. faecalis* obtained between treatment groups at each depth of dentine as well as the differences at the two different depths within each treatment group. The level of significance was set at 5%.

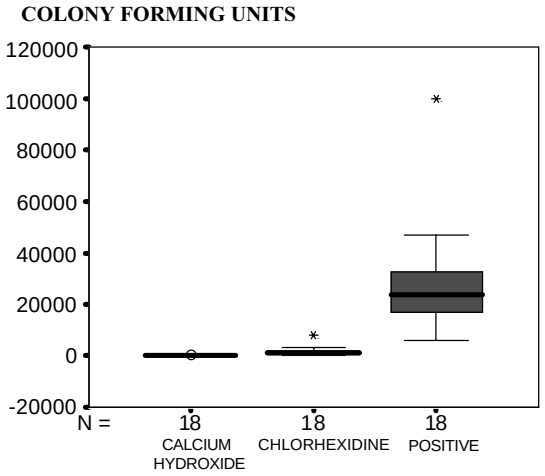

Figure 1 Box plot representing the number of CFU of *E. faecalis* present in groups A–C at 100 μm into dentine.

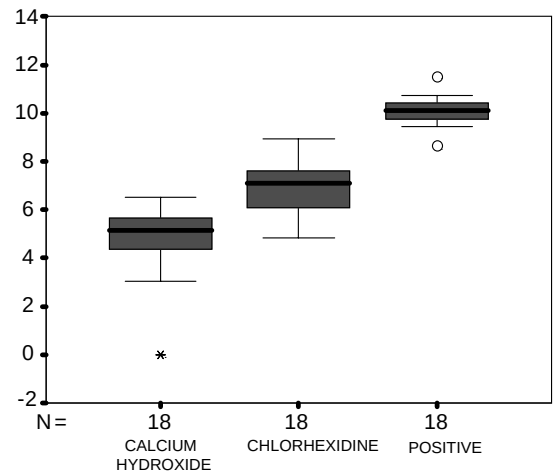

Figure 2 Box plot representing the number of CFU of *E. faecalis* present in groups A–C at 100 μm into dentine after log transformation.

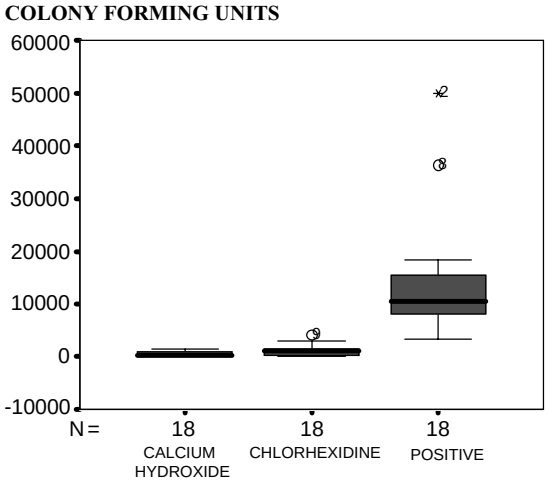

Figure 3 Box plot representing the number of CFU of *E. faecalis* present in groups A–C at 250 μm into dentine.

Results

All experimental samples inoculated showed purified inoculum of *E. faecalis* in all final plated samples. There was a trend to recover higher mean counts in the inoculation controls at dentine depths of 100 (1.33×10^5 CFU mL^{-1}) and 250 μm (6.85×10^4 CFU mL^{-1}), respectively, compared to the positive controls (group C) at the same dentine depths (2.92×10^4 and 1.28×10^4 CFU mL^{-1}).

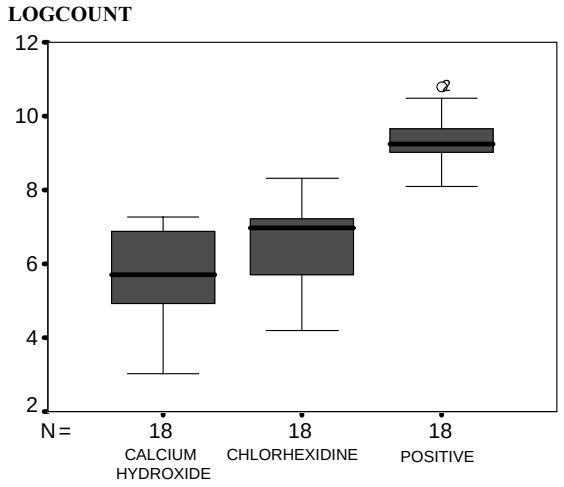

Figure 4 Box plot representing the number of CFU of *E. faecalis* present in groups A–C at 250 μm into dentine after log transformation.

The results of the *E. faecalis* counts (CFU) recovered from the dentine samples of groups A–C is presented in Table 2. At the 100 and 250-μm dentine depths, there were significantly fewer colonies isolated in group A treated with calcium hydroxide (2.1×10^2, 5.11×10^2) or group B with chlorhexidine 'activ points' (1.58×10^3, 1.11×10^3) as compared to the positive control group ($P < 0.05$). There was also a significant difference between the two treatment groups at a depth of 100 μm, with calcium hydroxide being significantly more effective than the chlorhexidine points in reducing the number of colonies of *E. faecalis* recovered ($P = 0.013$). This difference did not reach statistical significance for samples obtained at the depth of 250 μm ($P = 0.353$).

In the calcium hydroxide group, CFU detected was significantly less in superficial dentine (100 μm) compared to deeper layers (250 μm; $P = 0.000$). The reverse was noted for the positive control ($P = 0.000$), whereas there was no significant difference in the CFU detected at either depth of dentine for 'activ points' ($P = 0.101$; Table 2).

Discussion

The *in vitro* infected dentine model used in this experiment was modified from that developed by Haapasalo & Orstavik (1987) without the removal of cementum (Almyroudi *et al.* 2002). Human two-rooted maxillary premolars were selected with root lengths and diameters

Table 2 Statistical analysis of the number of CFU for the three treatment groups and between the two dentine depths

	Dentine depths								Dentine depths	
	100 μm			250 μm			Paired *t*-test	Paired *t*-test		
Group	Mean ± SD	Median	Range	Mean ± SD	Median	Range	between depths	between groups	100 μm	250 μm
I ($n = 5$)	132800 ± 69958	94000	66000–217500	68500 ± 46544	43000	25000–127500	–	–	–	–
A ($n = 18$)	210 ± 183	175	0–675	511 ± 452	305	20–1460	0.000	A and B	0.013	0.353
B ($n = 18$)	1581 ± 1836	1202	125–7850	1105 ± 1037	1055	65–4115	0.101	B and C	0.000	0.002
C ($n = 18$)	29210 ± 21081	23925	5600–100000	12830 ± 11504	9325	2800–50000	0.000	C and A	0.000	0.000

A, calcium hydroxide; B, 'activ points'; C, saline (positive control); I, inoculation control.
Level of significance set at 0.05.

appropriate for dentine sampling using rotary nickel–titanium files (Han *et al.* 2001). In this study, ProFile .04 series sizes 60 and 90 were able to sample dentine to depths of 100 and 250 μm, where a mean count of 1.33×10^5 and 6.85×10^4 CFU mL^{-1} of *E. faecalis* were recovered, respectively, under the inoculation controls. Scanning electron microscopic evaluation had earlier demonstrated a correlation between culturing from dentine samples and dentine tubule infection (Orstavik & Haapasalo 1990). The latter was facilitated by the removal of smear layer (Safavi *et al.* 1989, Love *et al.* 1996) using 17% EDTA and 5.25% sodium hypochlorite for 4 min each (Haapasalo & Orstavik 1987, Love 1996).

Enterococcus faecalis, ATCC 29212, was chosen as the test species because of its implication as a possible microbial factor in therapy-resistant apical periodontitis (Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001). It is a nonfastidious microbe that is relatively easy to culture and has been shown *in vitro* to predictably penetrate deeply into human dentinal tubules (Akpata & Blechman 1982). It is also suggested to be considerably resistant to calcium hydroxide (Stevens & Grossman 1983, Bystrom *et al.* 1985, Orstavik & Haapasalo 1990), which is the most commonly used intracanal medicament. This study compared the antimicrobial effect on infected dentinal tubules of intracanal chlorhexidine-impregnated gutta percha points ('activ points') and calcium hydroxide. Precautions to prevent dehydration of the latter, speculated to reduce its alkalinity (Almyroudi *et al.* 2002), was achieved by surrounding the root specimens with sterile saline-dampened gauze in individually closed Eppendorf tubes.

The results of this study indicated that calcium hydroxide was not able to completely eliminate *E. faecalis*, as evidenced by the counts recovered (Table 2). The relative inefficacy of calcium hydroxide against *E. faecalis* found in this study was in accordance with other studies on both bovine (Orstavik & Haapasalo 1990, Siqueira & Uzeda 1996) and human teeth (Safavi *et al.* 1990, Estrela *et al.* 1999). It was reported that intracanal calcium hydroxide reached inner peak dentine pH as high as 12.3 cervically (Ho *et al.* 2003) and 9.7 apically (Nerwich *et al.* 1993) within hours, whereas outer peak dentine pH of 9.3 cervically and 9.00 apically were achieved after 2–3 weeks (Nerwich *et al.* 1993). While most bacteria are readily susceptible to a pH of 9 (Harper & Loesche 1984), *E. faecalis* has been shown to survive at pH 11.5 or less (Bystrom *et al.* 1985). It was also noted in the calcium hydroxide group that there was significantly less CFU mL^{-1} of *E. faecalis* detected in the superficial dentine at 100-μm depth (2.10×10^2) compared to the deeper layers at 250 μm (5.11×10^2). These results appeared to suggest that microorganisms were exposed to lethal levels of hydroxyl ions only at the tubule orifice (Siqueira & Lopes 1999) and that the hydroxyl ions were increasingly buffered and impeded by diffusion with increasing dentine depth (Wang & Hume 1988, Nerwich *et al.* 1993) and, consequently, too overwhelmed to exert their lethal effect on *E. faecalis*.

Chlorhexidine has been shown to be effective against *E. faecalis in vitro* (Hennessey 1973). In this study, the antimicrobial effect of 'activ points' was similarly demonstrated (Table 2). As verified by the baseline control of gutta percha prior to the experiment proper, this was unlikely to be because of the high content of zinc oxide in the gutta percha exerting an antimicrobial effect (Moorer & Genet 1982, Podbielski *et al.* 2000). 'Activ points' were found to result in a 10-fold less count of *E. faecalis* (1.58×10^3 and 1.11×10^3 CFU mL^{-1}) compared to the positive controls (2.92×10^4 and 1.28×10^4 CFU mL^{-1}). These significant differences ($P = 0.000$ and 0.002) were possibly attributed to the large quantities of embedded chlorhexidine diacetate that initially dissociated and was gradually released from the surface of the 'activ points' upon contact with moisture. Presumably, the rate of release is dependent on the amount of fluid present in the canal available for dissociation. In this study, 5 μL of distilled water was added to the 'activ points' in the root canal, and the root specimens were thereafter kept moist to promote the dissociation kinetics. The fact that there was no significant difference ($P = 0.101$) in the CFU detected at the two depths of dentine sampled seemed to indicate that the antimicrobial effect of chlorhexidine, though present at a deeper depth of 250 μm, might be at too low an inhibitory concentration to achieve complete elimination of *E. faecalis*. The limited volume of fluid in the root canal might have hindered further drug release because of drug accumulation and the associated saturation (Huang *et al.* 2000). Gomes *et al.* (2001) found that 1% and 2% chlorhexidine gluconate took significantly less time to kill *E. faecalis* compared to 0.2% chlorhexidine, although all concentrations were eventually effective. It is not known if the antibacterial effect of 'activ points' found in this study would be improved if they had been left in the root specimens for a longer period of time.

As shown in Table 2, chlorhexidine 'activ points' were found to be significantly less effective compared to calcium hydroxide paste at a depth of 100 μm into dentine ($P = 0.013$), but at the deeper depth of 250 μm, the difference was not distinct ($P = 0.353$). It is not known if the better antimicrobial effect of calcium hydroxide was

because of its speculated ability to act as a physicochemical barrier depriving bacteria of nutrients and space to multiply or the difference in the formulations or both. Previous studies reported more favourable results with a gel formulation of chlorhexidine gluconate compared to similar concentrations in a sustained release device (Lenet *et al.* 2000, Barthel *et al.* 2002, Basrani *et al.* 2002). The better adaptability of the calcium hydroxide paste to the intricacies of the root-canal system might similarly allow for better diffusion. In contrast, only one 'activ point' with .02 taper was used in the inoculated root canal prepared to .04 taper. It is possible, however, speculative, if better adaptation would improve the antibacterial effect of 'activ points' in addition to the improved dissociation kinetics in the presence of replenished intracanal fluid.

The substantivity of chlorhexidine on hard tissue (Parsons *et al.* 1980) was a concern in this study, where the dentinal shavings were cultured to obtain bacterial counts. In order to avoid false-negative results, a combination of 0.3% L-α-lecithin and 3% Tween 80 was used (Zamany & Spangberg 2002). The former contains phosphatidylcholine that inactivates chlorhexidine, while the latter maintains the phosphatidylcholine in solution (Zamany & Spangberg 2002). The use of these chemicals has been found to have no detrimental effect on the recovery of test bacteria (Zamany & Spangberg 2002). Likewise, 0.5% citric acid was used as the inactivating agent for calcium hydroxide (Moller 1966). It was believed that 0.5% citric acid should have no bearing on the results as its antibacterial effect would be buffered by live bacteria (Shuping *et al.* 2000). In this study, inactivators were used (Bender & Seltzer 1954) rather than centrifuging and diluting in a large volume of culture medium (Vahdaty *et al.* 1993) to predictably eradicate any possible trace of residual antimicrobial agents.

Different methodologies have been employed as measures for bacterial growth. These include turbidity assessment of the broth containing incubated dentine shavings by optical density readings from a spectrophotometer (Heling *et al.* 1992, Komorowski *et al.* 2000, Lenet *et al.* 2000, Han *et al.* 2001, Basrani *et al.* 2002) or the incubated root specimens by visual inspection (Safavi *et al.* 1990). The present study adopted a quantitative measurement taking into account the viability of the microorganisms. After obtaining the dentine samples, *E. faecalis* was cultured and the number of colonies were quantified using the plate count technique (Vahdaty *et al.* 1993, Almyroudi *et al.* 2002). The observation that the mean counts obtained for the positive controls were lower than that from the 3-week inoculation controls seemed to imply that the withdrawal of nutrient support is detrimental to the survival of *E. faecalis* (Orstavik & Haapasalo 1990). However, at least 1.28×10^4 CFU mL^{-1} was still detected at a dentine depth of 250 μm. Enterococci have been shown to possess virulence factors that allow survival under harsh ecological conditions as a single organism (Fabricius *et al.* 1982, Love 2001). Although the consequences of dentine tubule infection and the possible role of the remaining bacteria in the dentinal tubules after root-canal treatment leading to long-term failure (Oguntebi 1994, Peters *et al.* 1995) have not been ascertained, the mainstay of root-canal treatment is still the elimination of bacteria. Therefore, measures should constantly be employed to search for alternative, more effective intracanal medications. Further studies with other clinical isolates of this bacterium are warranted.

Conclusions

Based on the current experimental design, the following conclusions could be made:

1 Chlorhexidine-impregnated points ('activ points') did not possess an inhibitory activity strong enough to completely eliminate a moderately large number of a single endodontic pathogen, *E. faecalis*, from infected human dentinal tubules.

2 The antimicrobial effect of chlorhexidine-impregnated points ('activ points') was less than that of the aqueous calcium hydroxide paste; however, this was not significant at the dentine depth of 250 μm.

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