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Antimicrobial activity of various 'active' gutta-percha points against *Enterococcus faecalis* in simulated root canals

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Abstract

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Aim To test the antimicrobial activity of various gutta-percha points against *Enterococcus faecalis* in simulated root canals.

Methodology Root canals were simulated by inoculated glass capillaries. A 2.5 μL increment of a suspension of *E. faecalis* was placed into 10 simulated root canals together with Calcium Hydroxide^R points (CHP), Calcium Hydroxide Plus^R points (CH+P), active points^R (AP), conventional gutta-percha points (CP) (Coltène Whaledent, Langenau, Germany) or no points (NP) (control) (each $n = 2$). The points and simulated root canals were flushed with 2 mL of sterile saline solution after 10 min or after 5 h of anaerobic incubation (37 °C, 100% humidity). Dilution sequences until 10^{-3} and 10^{-4} were prepared and plated on agar plates. The original suspension, diluted until 10^{-6} and 10^{-7}, served as another control. The numbers of colony forming units were counted after 24 h. This experimental procedure was repeated 15 times.

Results Without gutta-percha points, bacteria grew threefold in number within 5 h. With CHP and CH+P bacterial counts at 10 min and 5 h were approximately 50% compared with the control. AP killed all bacteria within 5 h. With CP, bacteria proliferated more than without points (counts at 5 h 177% of NP). Except for CHP versus CH+P differences between groups were statistically significant (Mann–Whitney test, $P < 0.01$).

Conclusions In this experimental model, the potential of CHP and CH+P to kill *E. faecalis* was limited. CP stimulated bacterial growth. AP killed all bacteria after 5 h.

Keywords: antimicrobial activity, active point^R, calcium hydroxide^R point, calcium hydroxide plus^R point, *E. faecalis*, simulated root canals.

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Introduction

Conventional intracanal medicaments are commonly used in a paste-form, which is not easy to manipulate (Staehle *et al.* 1997) and difficult to remove completely from the root canal system (Ricucci & Langeland 1997, Lambrianidis *et al.* 1999, Sevimay *et al.* 2004). In response to these potential difficulties, sustained releasing devices were developed (Heling *et al.* 1992a,b), which can easily be removed at the second appointment. Such 'active' gutta-percha points contain substances with antimicrobial activity that are released as soon as moisture touches the surface (Distler & Petschelt 1997). Calcium Hydroxide^R points (CHP) showed good antimicrobial activity against *Streptococcus oralis* H1 and *Actinomyces naeslundii* A65 within simulated root canals (Brüß 1998). Podbielski *et al.* (2000) evaluated points with additions of calcium hydroxide, zinc oxide (ZnO), a mixture of ZnO and chlorhexidine (ChX), ZnO and iodine-polivinylpyrroli-

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dine (J-PVP) or a mixture of ZnO, ChX and J-PVP. The growth of the bacteria was inhibited by the gutta-percha points to a different extent. From all microorganisms tested, *Peptostreptococcus micros* and *Enterococcus faecalis* were least susceptible to the antimicrobial agents. In another study, *E. faecalis* proved to be resistant against gutta-percha points containing iodoform (Shur *et al.* 2003).

The aim of the present study was to evaluate the antibacterial activity of calcium hydroxide- or ChX-releasing gutta-percha points to *E. faecalis* in simulated root canals. The null hypothesis was that neither the type of gutta-percha points nor the time influenced the bacterial counts or the growth rate.

Materials and methods

Simulated root canals

Small glass capillariy tubes, made of Pasteur Pipettes (Chase Instruments Corporation, Glens Falls, NY, USA) were separated in a region where a size 90 gutta-percha point with a 0.02 taper touched the walls. The narrower ends of the capillaries were sealed over a Bunsen burner to create small 'test tubes' (see Fig. 1a), and into which gutta-percha cones could be placed (see Fig. 1b). The radii of the tubes were approximately 0.5 mm and the lengths approximately 23 mm resulting in a capacity of approximately 28 μL, so that 2.5 μL of fluid could be applied together with the gutta-percha point without overflow. The tubes acted as simulated root canals.

Microorganism

To grow *E. faecalis* NCIB 8191, 100 μL of bacterial suspension was pipetted into a sterile test tube containing 10 mL of sterile Schaedler-Broth (Difco, Detroit, MI, USA). The bacteria were incubated for 16 h at 37 °C under anaerobic conditions (with Gas Pak^R; Becton Dickinson, Cockeysville, MD, USA). Growth curves were carried out and measured with a Photometer (UV240; Shimadzu, Kyoto, Japan) at 620 nm as well as by performing a dilution sequence, plating on Agar plates (made with Schaedler Broth) and counting the colony forming units (CFU).

Gutta-percha points

For the main investigation, the bacteria were used in the exponential growth phase after 3 h of incubation. Ten sterile simulated root canals were incubated with 2.5 μL of bacterial suspension (Fig. 1a) each. Two simulated root canals per group were filled with different gutta-percha points: CHP, Calcium Hydroxide plus^R points (CH+P), active points^R (AP, containing chlorhexidine diacetate), conventional gutta-percha points (CP, containing zinc oxide) (all size 90 points with 0.02 taper, manufactured by Coltène/Whaledent, Langenau, Germany) or left without gutta-percha point (NP, control) (Fig. 1b). All gutta-percha points were used 'straight out of the box', and care was taken to avoid their contamination. Thus, a further control group with a suspension without bacteria was not carried out.

From one simulated root canal per group the bacterial suspension was recovered immediately, which resulted in a contact time of the bacteria to the point of approximately 10 min. The other simulated root canals were incubated under anaerobic conditions (Gas Pak^R) for 5 h at 37 °C.

To recover the bacterial suspension, the closed ends of the glass capillaries were removed (Fig. 1c) and the glass capillaries washed with 1 mL (NP: 2 mL) of 0.9% sterile NaCl solution each (Fig. 1d). The gutta-percha points were also washed with 1 mL of 0.9% sterile NaCl solution each to recover as many bacteria as possible (Fig. 1e). The resulting 2 mL per test tube were collected in falcon tubes (Fig. 1f) and homogenized by a Branson Sonifier 250 (Heinemann, Schwäbisch Gmünd, Germany). A dilution sequence was completed up to 10^{-3} and 10^{-4} and plated on agar plates. As another control, 1 mL was taken from the original bacterial suspension at 10 min and a dilution sequence up to 10^{-7} was carried out. The last two dilutions were also plated on agar plates. The experiments were carried out under a laminar flow bank. All plates were read with a cell counting pen after 24 h of incubation at 37 °C under anaerobic conditions. The number of CFU was calculated. This experimental procedure was repeated 15 times, so that each group consisted of a total of 30 : 15 samples recovered after 10 min and 15 samples recovered after 5 h of incubation.

The following calculations were carried out: CFU at 10 min/5 h divided through the CFU of the corresponding original suspension ('absolute growth', representing the growth compared with the starting point); CFU at 10 min divided through CFU of NP at 10 min/CFU at 5 h divided through CFU of NP at 5 h ('relative growth', representing the influence of the gutta-percha point under test); CFU at 5 h divided through CFU of the same group at 10 min ('growth

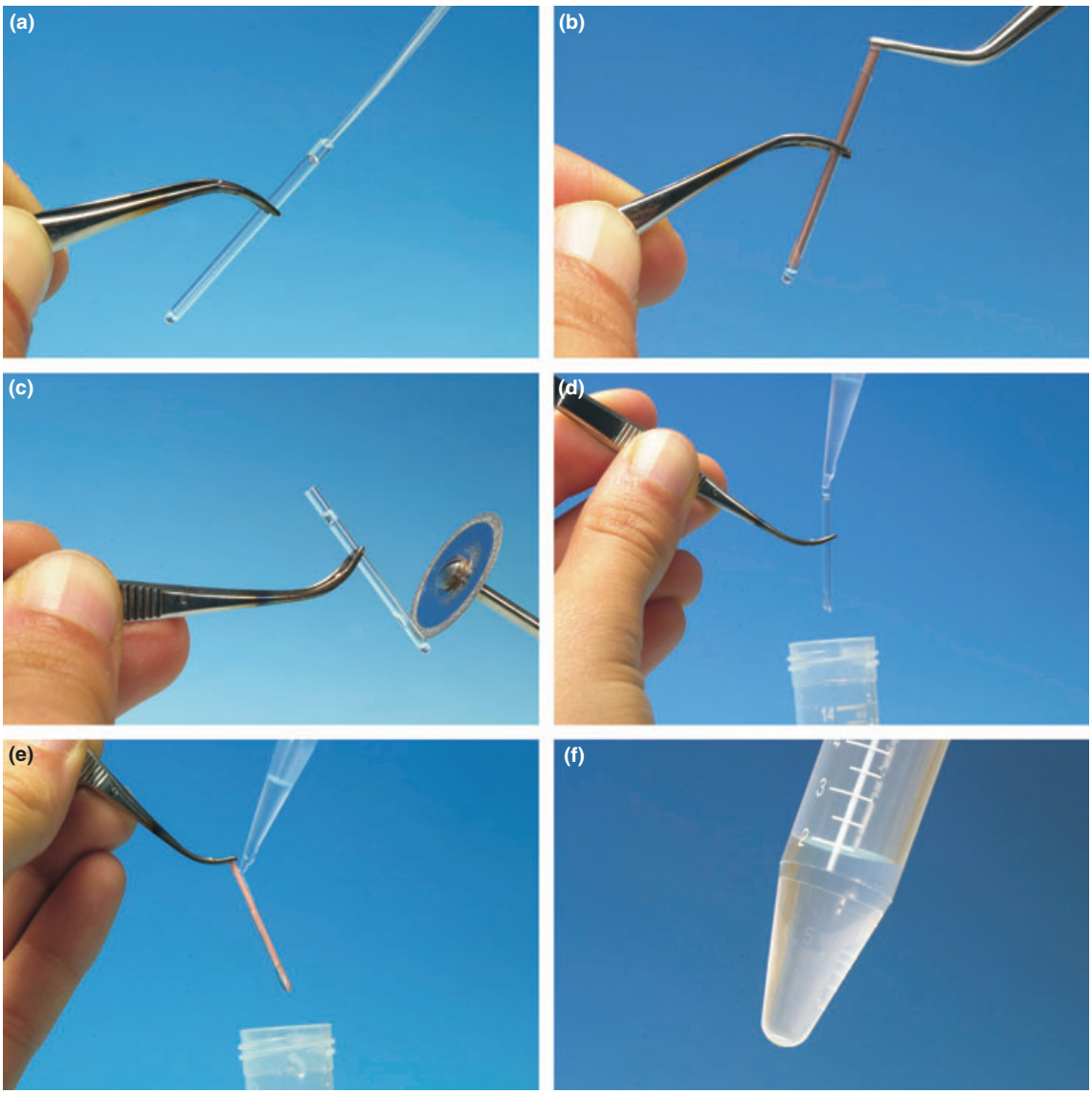

Figure 1 Simulated root canals in the shape of small test tubes were filled with 2.5 μL of bacterial suspension (a) and a gutta-percha point (b). Bacteria were recovered by cutting off the bottom of the glass capillary (c), removing the gutta-percha point and washing the capillary (d), washing the gutta-percha point (e) and collection of the suspension in a falcon tube (f).

rate', representing the growth between 10 min and 5 h within each group). Statistical tests (Kolmogorov-Smirnov tests, Kruskal–Wallis tests, Mann–Whitney tests, Wilcoxon tests) were carried out using SPSS^{R} WIN 12 (SPSS, Chicago, IL, USA). The level of significance was set at $\alpha = 0.05$.

Results

The CFU for different original suspensions within the fifteen different experimental procedures ranged from 1.85×10^8 to 9.6×10^8 (mean: 4.4×10^8, SD 2.3×10^8) so that 2.5 μL of bacterial suspension contained from 4.625×10^5 to 2.4×10^6 bacteria.

Kolmogorov-Smirnov tests revealed that some of the groups were not normally distributed ($P < 0.05$). Thus, non-parametric tests were used. Within the initial measurements without gutta-percha points (NP), virtually the whole amount of bacteria could be recovered (99.5%) (Fig. 2, NP at 10 min). With gutta-percha points present, the recovery rate varied from 0.2% (AP) to 86.9% (CP) (Fig. 2). Without points, bacteria grew

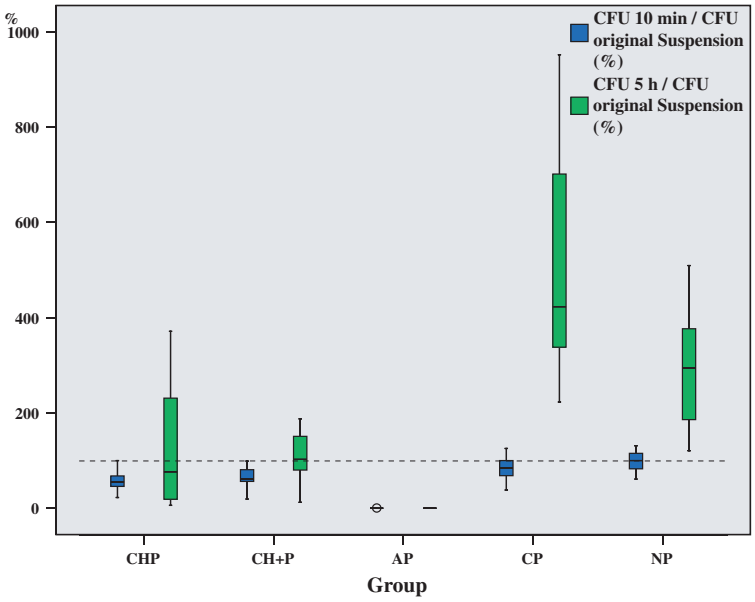

Figure 2 Absolute growth: Box plots depicting the CFU of *E. faecalis* related to CFU of the original suspension in %. Horizontal bars: medians; boxes: interquartile areas; error bars: 10th and 90th percentile; dots: extreme values. Dotted horizontal line at 100%. CHP: calcium hydroxide^{R} points; CH+P: Calcium Hydroxide Plus^{R} points; AP: active points^{R}; CP: conventional gutta-percha points; NP: no point (control); CFU: colony forming units. Within each group values at 5 h and 10 min were significantly different (Wilcoxon-tests, $P < 0.05$).

threefold in number within 5 h (Figs 2 and 4). After a contact time to AP of 5 h, all bacteria in all samples were killed (Figs 2 and 3). Although approximately half of the bacteria were killed in the CHP and CH+P groups at 10 min, bacterial growth could be detected after 5 h (Fig. 4). The growth rate for CH+P was significantly lower than for NP (Mann–Whitney test, $P < 0.05$). For CHP, no significant difference to NP could be found (Fig. 4). In contact with CP, an enhanced growth (177%) of *E. faecalis* could be detected compared with NP (Mann–Whitney test, $P < 0.001$) (Fig. 3). The results are also shown in Figures 2–4.

Statistical analysis revealed significant differences between groups for the bacterial counts relative to the original suspension ('absolute growth'), the counts relative to NP ('relative growth') and for the 'growth rate' (Kruskal–Wallis tests, $P < 0.001$). Mann–Whitney tests ($P < 0.01$) showed significant differences for 'absolute' and 'relative growth' between all pairs of groups, except for CHP and CH+P at 10 min and 5 h

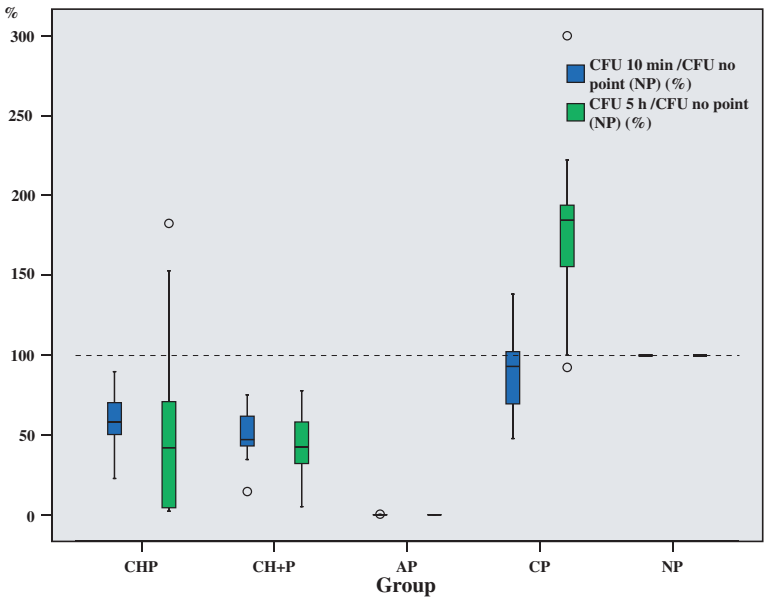

Figure 3 Relative growth: Box plots depicting the CFU of *E. faecalis* related to CFU of the corresponding sample without point (NP) in % (5 h compared with 5 h NP; 10 min compared with 10 min NP). Dotted horizontal line at 100%. CHP: calcium hydroxide^{R} points; CH+P: calcium hydroxide plus^{R} points; AP: active points^{R}; CP: conventional gutta-percha points; NP: no point (control); CFU: colony forming units. All possible pairs of groups were significantly different (Mann–Whitney tests, $P < 0.01$), except CHP and CH+P at 10 min and 5 h and CP and NP at 10 min.

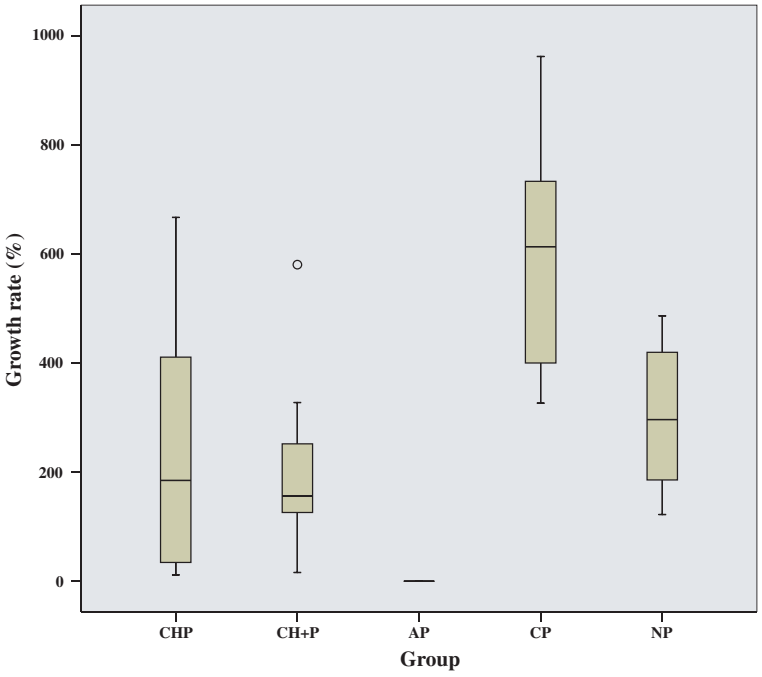

Figure 4 Growth rate: Box plots for CFU of *E. faecalis* at 5 h related to CFU of the corresponding sample at 10 min in %. CHP: calcium hydroxide[R] points; CH+P: calcium hydroxide plus[R] points; AP: active points[R]; CP: conventional gutta-percha points; NP: no point (control); CFU: colony forming units. All possible pairs of groups were significantly different (Mann–Whitney tests, $P < 0.05$) except CHP/NP and CHP/CH+P.

and CP and NP at 10 min. Wilcoxon tests revealed significant differences between 5 h and 10 min for all groups ($P < 0.05$) except CHP.

Discussion

Significantly different values for the bacterial counts could be found for different groups as well as for different time-points. Thus, the null hypothesis was rejected. Several tests can be used for the evaluation of antimicrobial activity. Agar diffusion tests are relatively easy to carry out, but testing of calcium hydroxide is problematic, because agar plates act as an effective buffer for the released hydroxyl ions because of their composition. In another test model (Haapasalo & Ørstavik 1987), the canals of root sections of bovine teeth are inoculated with bacteria and allowed to grow into the dentinal tubules (Ørstavik & Haapasalo 1990). Intracanal medicaments can be placed and the viability of the bacteria quantified within ground dentine close to the root canal.

In previous tests using natural teeth some difficulties were encountered when they were used for a full quantitative model of an infected root canal (Brüß 1998). Bacteria can grow into the dentinal tubules and through the foramen and therefore cannot be recovered. Thus, a simple root canal model, consisting of glass capillaries, was chosen. In an earlier study, it was shown to be suitable for the examination of the antimicrobial activity of solid bodies under spatial conditions that come close to that within the root canal (Brüß 1998).

In the present test model, the recovery rate of the bacteria without gutta-percha cone was 99.5% and with a conventional cone containing zinc oxide it was 86.9%. This means that with the use of conventional gutta-percha cones, some of the vital bacteria were lost between placement and removal of the bacterial suspension. This may be because of some initial antibacterial activity of CP or, more likely, to the porous surface structures of the cones. Despite this, the recovery rate of approximately 90% allows almost full quantitative measurements, so that this relatively easy to use model can serve as a screening method when developing or evaluating new materials to serve as intracanal medicaments. For this purpose, a 'control point', made of an inert material with the same size and shape like the solid body to be tested has still to be developed.

Within the production process, the gutta-percha points used are made in a fully automated manner. The manufacturer claims that 10 points contain approximately one bacterium. To check this claim the following procedure was carried out: from each of the packages six gutta-percha points were removed with sterile tweezers and rolled on blood agar plates which

were incubated for 72 h at 37 °C/100% humidity. In one case a single colony of coagulase-negative staphylococci was found. The claim of the manufacturer (production sterility) was confirmed (one bacterium on 24 points). Because of this, no further negative control without bacteria in suspension was carried out.

E. faecalis can grow in NaCl solutions with concentrations up to 6.5% and at a pH of 9.6. These growth characteristics are used to differentiate *E. faecalis* from streptococci (Sneath *et al.* 1986). *E. faecalis* lives in the intestine of humans and most animals and even in plants (Sneath *et al.* 1986) and thus can be looked at as ubiquitous. *E. faecalis* is more frequently found in cases of endodontic retreatments than in primary root canal treatment (Peciuliene *et al.* 2000, 2001, Hancock *et al.* 2001, Chávez de Paz *et al.* 2003, Pinheiro *et al.* 2003, Adib *et al.* 2004), and that may be due to its ability to survive time-periods of starvation (Figdor *et al.* 2003) of up to 12 months (Sedgley *et al.* 2005). Thus, it is an important microorganism in endodontics (Portenier *et al.* 2003), and was therefore chosen for the present study.

It has been shown that bacteria within the root canal form biofilms (Sen *et al.* 1999, Nair *et al.* 2005). This is important, because as biofilm phenotype, bacteria can be protected from antimicrobial agents by the matrix layer of the biofilm and by a reduced growth rate and thus are less susceptible to antimicrobial agents than as planctonic phenotype (Donlan & Costerton 2002). Regarding the susceptibility of a monospecies biofilm of *E. faecalis*, the literature reveals different results. *E. faecalis* within biofilms can be killed by calcium hydroxide suspension (Chai *et al.* 2007) or 2% chlorhexidine solution (Lima *et al.* 2001) when the biofilm is grown on membranes. When grown on dentine, *E. faecalis* biofilms are able to form a calcified surface layer (Kishen *et al.* 2006), especially under nutrition-deprived conditions (George *et al.* 2005) and can grow in the presence of calcium hydroxide medicaments (Distel *et al.* 2002). The situation within multi-species biofilms is even more complex, because different bacterial species can exchange resistance genes (Weigel *et al.* 2007). For future examinations an experimental model using mono- or multispecies biofilms including mineralized surfaces should be developed to obtain results that are more relevant to the clinical situation.

At 10 min the bacterial counts for both CHP were lower than for NP. This can be attributed to an initial antibacterial activity, as the release of calcium hydroxide out of the points is noticeable within a few minutes (Lohbauer *et al.* 2005). During 5 h, CHP allowed a growth rate that was not significantly different from that of NP, whereas for CH+P, a significantly lower growth rate (Mann–Whitney test, $P < 0.05$) compared with NP was found (see Fig. 4). This difference between both types of points can be attributed to the increased release rate for ions shown for CH+P (Lohbauer *et al.* 2005). On the other hand, both types of points offer a reduction in bacterial counts compared with NP of only approximately 50% (see Fig. 3). This seems rather low, as it is known that both types of Calcium Hydroxide points release a pH of approximately 12 (Lohbauer *et al.* 2005) and *E. faecalis* can only withstand values up to pH 11.0 (McHugh *et al.* 2004). This discrepancy may be explained by a possible inactivation of calcium hydroxide by died bacterial cells, so the remaining bacteria survive and proliferate. Portenier *et al.* (2002) found a similar inactivation of heat-killed cells of *E. faecalis* and *C. albicans* on the antimicrobial action of chlorhexidine digluconate and iodine potassium iodine.

Overall, the calcium hydroxide released from the points restricted bacterial growth to a limited extent. In the present model the bacteria come in direct contact to the points. The antibacterial activity *in vivo* is expected to be even more limited, because it can be impaired by different agents such as bovine serum albumin, collagen or by the buffering action of the dentine itself (Camps & Pashley 2000, Haapasalo *et al.* 2000, Portenier *et al.* 2001, 2002, 2006). Within the limits of this laboratory study, it can be concluded that both CHP would not be effective against *E. faecalis* within the clinical situation. Further tests including biofilm methods of growth are needed to clarify this issue.

For 'active points', the bacterial counts after 10 min were 0.2%, the counts of 5 h 0% compared with NP. This may be due to the rapid release of ChX out of the points. These findings are in agreement with previous studies, showing the effectivity of ChX as a possible alternative to calcium hydroxide as intracanal medicament in cases, where *E. faecalis* is suspected (Gomes *et al.* 2003, Menezes *et al.* 2003, Sirén *et al.* 2004). On the other hand, Öztan *et al.* (2006) found a growth inhibiting effect of AP that was similar to CH+P. In their study, some *E. faecalis* could survive in the presence of medicated points for up to 14 days. Differences in results may be attributed to the different amount of bacterial suspension used (200 vs. 2.5 μL) per medicated point and the different number of bacteria per mL. In another study (Barthel *et al.* 2002), active points were less effective than chlorhexidine gel in disinfecting root canals infected by human oral flora.

In the present experimental model, ChX came in direct contact with the bacteria, which were in an optimum growth phase. More studies have to be carried out to clarify, whether the antibacterial activity of AP is sufficient to work against *E. faecalis* within dentinal tubules and when in starvation mode. Experiments of Portenier *et al.* (2005) have proved that in this state the bacteria are difficult to kill.

Despite being less effective compared with the newly developed 'active' gutta-percha points (Podbielski *et al.* 2000), CP did reveal some limited antimicrobial activity, which can be attributed to their content of zinc oxide (Moorer & Genet 1982, Weiger *et al.* 1993). In this context, the results for CP were unexpected. The growth rate was nearly double, compared with the group without cones. Two reasons for these findings can be suggested: a growth-stimulating effect of the zinc oxide concentration released from the points or a stimulating effect because of the bigger contact surface to the bacterial suspension. As a third possible explanation, the forming of biofilms is not likely because of the relatively short time for growth and the lack of serum within the bacterial culture medium. The former is needed for *E. faecalis* to form biofilms on gutta-percha (Takemura *et al.* 2004). This growth stimulating effect, found in the present study, has to be clarified by further experiments.

Conclusion

Both types of Calcium Hydroxide points revealed only slight antibacterial activity against *E. faecalis*; CP stimulated its growth. 'AP' killed all bacteria after 5 h.

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