doi:10.1111/j.1365-2591.2006.01173.x

A comparative study on the disinfection potentials of bioactive glass S53P4 and calcium hydroxide in contra-lateral human premolars *ex vivo*

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Abstract

Zehnder M, Luder HU, Schätzle M, Kerosuo E, Waltimo T. A comparative study on the disinfection potentials of bioactive glass S53P4 and calcium hydroxide in contra-lateral human premolars *ex vivo*. *International Endodontic Journal*, **39**, 952–958, 2006.

Aim To evaluate the effects of bioactive glass S53P4 versus calcium hydroxide when used as dressings in contra-lateral human premolars infected with *Enterococcus faecalis* ATCC 29212.

Methodology Pairs of contra-lateral premolar teeth plus single control premolars were obtained from 23 individuals aged 10–26 years undergoing orthodontic treatment. Root canals of teeth with fully formed apices (nine contra-lateral pairs, seven controls) were instrumented using a size 60 FlexoFiles 2 mm short of canal length. Canals with open apices (six contra-lateral pairs, four controls) were circumferentially instrumented using a FlexoFile. Root canals were rinsed with 1% sodium hypochlorite and 10% citric acid. Teeth were then suspended in tryptic soy broth (TSB) and autoclaved. Positive controls and study teeth were infected with *E. faecalis* ATCC 29212 for 2 weeks in TSB, while negative controls were kept in sterile TSB. Subsequently, contra-lateral premolars were dressed with bioactive glass S53P4 (BAG) or calcium hydroxide suspensions for 10 days. Dentine samples were obtained from teeth with fully formed apices using ISO-size 70, 80 and 90 FlexoFiles to working length and cultured. Teeth with open apices were fixed, fractured and examined using scanning electron microscopy (SEM).

Results Calcium hydroxide had a strong antibacterial effect and was significantly more effective than BAG in preventing residual bacterial growth ($P < 0.01$). SEM analysis revealed apparent substance-specific modes of action.

Conclusions Calcium hydroxide was an effective disinfectant in human teeth.

Keywords: bioactive glass, calcium hydroxide, disinfection, *Enterococcus faecalis*.

Received 12 April 2006; accepted 5 June 2006

Introduction

Calcium hydroxide has long been recommended for the treatment of 'fistula dentalis' (Nygren 1838). Recent clinical studies, however, have raised doubt over the efficacy of calcium hydroxide as an intracanal dressing (Peters *et al.* 2002, Waltimo *et al.* 2005). *Ex vivo* investigations using either direct exposure tests in the presence of dentine powder or infected bovine dentine blocks have further challenged the status of calcium hydroxide as the ideal antimicrobial dressing (Portenier *et al.* 2001, Zehnder *et al.* 2003). However, *ex vivo* observations cannot necessarily be extrapolated to the *in situ* situation in human teeth. In direct exposure tests, saturated calcium hydroxide solutions rather

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than aqueous suspensions were used; the former are much less effective than the latter (Stevens & Grossman 1983). Bovine dentine blocks contain a wider pulp space, wider dentinal tubules and more open tubules than human teeth (Paqué *et al.* 2006). Consequently, most *ex vivo* studies have used galenic calcium hydroxide preparations and/or medication-to-dentine ratios, which do not reflect the clinical reality. In comparative studies in human teeth, on the other hand, aqueous calcium hydroxide suspensions have usually performed better than or equally well as control materials (Barthel *et al.* 2002, Zerella *et al.* 2005).

Despite their limitations, the relatively simple *ex vivo* models mentioned above appear useful to screen a wide variety of antiseptic materials for their possible use in infected root canals. Such pilot studies have recently yielded interesting data on a SiO_2–Na_2O–CaO–P_2O_5 bioactive glass. An aqueous bioactive glass S53P4 suspension disinfected bovine dentine blocks more efficiently from an *Enterococcus faecalis* infection than a corresponding calcium hydroxide suspension (Zehnder *et al.* 2004). In direct exposure tests with aqueous suspensions of S53P4, the presence of dentine powder triggered an increased glass dissolution, which resulted in elevated pH and silica levels, rendering the glass more bactericidal (Zehnder *et al.* 2006). This bioactive glass was the first material ever tested in such a setting that showed positive interaction with dentine.

Based on these promising early results, the aim of the current investigation was to assess the antibacterial efficacy of bioactive glass in extracted human teeth. In a comparative setting, infected contra-lateral premolars were dressed with either aqueous calcium hydroxide or bioactive glass suspensions. After 10 days of incubation, root dentine samples were obtained and cultured. A further set of premolars was used to visually assess dentine infection and modes of action of the medications under investigation using scanning electron microscopy (SEM).

Materials and methods

Experimental teeth and instrumentation procedures

Teeth used for this study were premolars extracted for orthodontic reasons. All the teeth were free of caries and restorations. The current research protocol did not alter the treatment plan of the patients, who gave informed consent that their extracted teeth could be used for study purposes. The institutional ethics committee approved the procedures. Eighteen contra-lateral premolars with fully formed apices were obtained from nine individuals 13–24 years of age (median = 16 - years). These teeth were used for the assessment of remaining microbial growth after dressing of infected teeth with either bioactive glass or calcium hydroxide (see below). An additional 12 contra-lateral premolars with open apices from six individuals aged 10–14 years of age (median = 12 years) were used for SEM observations, and 11 premolars from eight individuals aged 15–26 (median = 17 years) as negative and positive controls (Table 1). After extraction, teeth were kept at −25 °C in individual tight containers. Personnel handling the specimens used universal precautions, i.e. they wore latex gloves, protection goggles and face masks.

Table 1 Control premolars used in this study

Type of control	*n*	Treatment
(+) Microbiology	4	Instrumentation, infection, incubation, root dentine sampling
(–) Microbiology	3	Instrumentation, incubation, root dentine sampling
(+) Histology	3	Instrumentation, infection, incubation, fixation, fracture, SEM
(–) Histology	1	Instrumentation, incubation, fixation, fracture, SEM

Before instrumentation, teeth were thawed at room temperature and immersed in 5% (w/v) sodium hypochlorite for 10 min to dissolve organic tissue remnants from the root surfaces. Any remaining soft tissue was removed using a Gracey curette. Radiographs of the experimental teeth were taken in a mesio-distal direction to assess root canal anatomy. After gaining access to the root canal system with a diamond-coated bur, canals were instrumented using ProFile (Dentsply Maillefer, Ballaigues, Switzerland) instruments size 60,.04 taper to size 45,.04 taper in a crown-down manner to two-thirds of the expected working length. A size 10 K-File (Dentsply Maillefer) was inserted into the root canal until the tip was just visible beyond the foramen and the working length determined by subtracting 2 mm from this length. An apical stop was created at this level using ProFile size 45,.04 taper instruments at working length. Apical enlargement to a size 60,.02 taper was then performed using hand instruments (FlexoFiles, Dentsply Maillefer). During instrumentation root canals were irrigated with a total of 10 mL of a 1% NaOCl solution using a 30-gauge irrigating needle (Hawe Neos, Bioggio, Switzerland). After instrumentation all root canals were rinsed with 10 mL of 10% citric acid to stop the NaOCl action and

remove the smear layer (Zehnder *et al.* 2005), followed by copious amounts of distilled water.

Teeth with open apices for SEM analysis were accessed and irrigated as described above, but instrumented by circumferential filing using a size 60 hand instrument (FlexoFile, Dentsply Maillefer).

Infection and disinfection

Experimental and control teeth were rinsed with 2 mL tryptic soy broth (TSB, Oxoid Ltd, Hampshire, UK) using a 30-gauge needle (Hawe Neos). Subsequently, teeth were placed in individual glass tubes containing 5 mL TSB. Test tubes containing the teeth were covered with metallic caps and autoclaved for 15 min at 121 °C. The tubes were then sonicated in a water bath for 5 min at room temperature. All tubes except those containing negative control teeth were seeded with 0.1 mL of overnight cultures of *E. faecalis* ATTC 29212 in TSB spectrophotometrically adjusted to 10^7 cells mL^{-1}. Tubes were incubated in ambient air at 37 °C for 2 weeks. The broth was aseptically changed at 2- to 3-day intervals. Purity of the culture was checked after 1 and 2 weeks of incubation by cultivation on tryptic soy agar and subsequent observation of colony morphology as well as cellular characteristics after Gram staining. This and all the subsequent laboratory procedures were exercised under aseptic conditions in a microbiological safety cabinet (SFE.120 EN; SKAN AG, Basel, Switzerland).

All teeth were then rinsed with 2 mL of sterile saline solution. Contra-lateral premolars were dressed with a sterile suspension of either calcium hydroxide powder (Merck, Darmstadt, Germany) or bioactive glass S53P4 (BAG, S53P4 – LOT 604; Vivoxid, Turku, Finland) in water. The BAG powder had a particle size of <45 μm; it was the same material that had been used in a previous study on bovine dentine block disinfection (Zehnder *et al.* 2004). The powder to liquid ratio for both materials was chosen so that thin slurries were obtained, which could be transported into the root canals using a lentulo spiral (Dentsply Maillefer) in a contra-angle hand piece (KaVo, Biberach, Germany). The solid/liquid ratio (w/v) of BAG/H_2O was 1/0.6; the corresponding ratio for $Ca(OH)_2$ 1/1.5. As both materials apparently exert their antimicrobial action through the steady release of ions in aqueous solution (Proell 1949, Zehnder *et al.* 2006), access cavities were dried with compressed air, but canals were left wet. Root canals were completely filled with the materials. Positive and negative control teeth were not medicated. All teeth were then transferred to individual sterile microcentrifugation tubes containing a wet cotton pellet soaked in 300 μL of water. By closing the lid of the tubes, a 100% humid environment could be maintained. Specimens were incubated at 37 °C for 10 days.

Harvesting of dentine samples from premolars with fully formed apices

Before sampling of dentine chips, teeth were irrigated with 2 mL of sterile saline solution. Subsequently, dentine was cut in the apical area of the canal applying the balanced force technique (Roane *et al.* 1985) at working length using a size 70 followed by an 80 and finally a 90 hand instrument (FlexoFile, Dentsply Maillefer). The files were separated 5 mm from the tip using a sterile wire cutter. File tips were directly transferred to sterile glass tubes containing 5 mL TSB. In teeth with two canals, dentine chips were obtained from only one canal. To prevent contamination from the outer tooth surfaces, the crowns of the teeth were briefly exposed to a gas flame. Tubes containing the files were incubated up to 5 days to detect growth of *E. faecalis*. Purity of growth was assessed as described above.

Microscopic evaluations on premolars with open apices

Half of the specimens with an open apex were irrigated with 2 mL of a fixation solution (half-strength Karnovsky's fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.02 mol L^{-1} sodium cacodylate buffer, pH 7.2) using a 30-gauge irrigating needle to rinse out the dressing materials. The other specimens were directly placed in the fixative. Specimens were fixed for 2 days at 5 °C. Longitudinal grooves, which did not penetrate into the canal, were then placed in the buccal and lingual surfaces of the teeth to facilitate their fracture. Teeth were fractured along these grooves after dipping them in water and then in liquid nitrogen. Subsequently, specimens were washed three times in a sodium cacodylate buffer (0.185 mol L^{-1}), and then immersed in 1% osmium tetroxide in 0.1 mol L^{-1} sodium cacodylate buffer for 2 h at room temperature. Thereafter, tooth halves were dehydrated using an ascending acetone series and dried using the critical point method in an CPD 030 device (BAL-TEC, Balzers, FL, USA). Dry specimens were glued to SEM stubs and sputter-coated with platinum in an MED 010 apparatus

(BAL-TEC). They were examined in a Tescan VEGA TS 5316 XM scanning electron microscope (Brno, Czech Republic) at an accelerating voltage of 20 kV and 10–15 mm working distance. Digital images were taken at a resolution of 2048 × 1536 pixels.

Data analysis

Qualitative data pertaining to persisting bacterial growth from root canals of contra-lateral premolars dressed with either BAG or calcium hydroxide were compared using chi-square tests.

Results

Microbiology (premolars with fully formed apices)

Negative controls, i.e. teeth that were subjected to all preparation steps save the mono-infection with *E. faecalis* ATCC 29212, remained free of bacterial growth. Positive controls showed vigorous growth of *E. faecalis* from size 70 to size 90 instruments. In the nine pairs of contra-lateral premolars, the calcium hydroxide suspension eradicated cultivable bacteria in all but one tooth, from which the dentine sample obtained with the size 90 instrument was positive. In contrast, the BAG suspension had only a mild antimicrobial effect. All but one of the nine premolars dressed with BAG showed some growth in at least one of the three sampled layers (Table 2). The difference in antimicrobial efficacy between calcium hydroxide and BAG was significant below the 0.01 level in all three sampled layers. All control cultures during the infection and the samples after disinfection procedures showed pure growth of *E. faecalis*.

SEM observations (premolars with open apices)

The dentine walls of positive controls showed clusters of coccoidal bacteria. Strings of these cells were observed in some dentinal tubules penetrating up to 100 μm towards the periphery; however, the great majority of tubules had remained uninfected. In contra-lateral premolars that were not irrigated with the histologic fixation solution after the experimental period, dentine walls were completely covered with the dressing materials. The BAG was fairly coherent and appeared to adhere to the dentine, as it remained in the root canals after fracturing and fixation, while the bulk of the calcium hydroxide was washed out during that process. In the specimens that were rinsed with the fixative, medication-specific observations were made on the bacteria covering the dentinal walls. Some bacteria subjected to a calcium hydroxide dressing had lost their cellular integrity, while others appeared intact (Fig. 1). In premolars dressed with BAG individual bacteria seemed to exhibit a calcified surface, while the majority looked intact. Generally, more bacteria were detected in teeth dressed with BAG than in counterparts medicated with calcium hydroxide. Dentine walls also looked different in the two groups. In the calcium hydroxide group, the walls appeared similar to the corresponding walls in the control teeth, except for some remaining material particles. In the BAG group, on the other hand, dentine walls were covered with small needle-like structures, which apparently did not stem directly from the dressing material (Fig. 1).

Table 2 *Enterococcus faecalis* ATCC 29212 recovery from dentine samples of mono-infected contra-lateral premolars after root canal dressing with either $Ca(OH)_2$ or bioactive glass S53P4 suspensions for 10 days

Sampling instrument	Positive growth $Ca(OH)_2$	Positive growth S53P4	χ^2	*P*-value
ISO size 70	0/9	6/9	9	<0.01
ISO size 80	0/9	7/9	11.46	<0.001
ISO size 90	1/9	8/9	10.89	<0.001

Discussion

In contrast to the results of a previous study using bovine dentine blocks infected with *E. faecalis*, the current *ex vivo* study revealed a superior disinfecting capacity of a calcium hydroxide compared with a bioactive glass S53P4 suspension in contra-lateral human teeth.

Contra-lateral human premolars extracted for orthodontic reasons represent an ideal *ex vivo* set-up for comparative material analyses, as they are anatomically almost identical (Wood & Green 1969). Consequently, bias caused by differences in root canal anatomy or dentine structure (Vasiliadis *et al.* 1983) can be excluded. An *E. faecalis* strain was used in the present study to infect root canal systems, as enterococci have been associated with persisting and recurrent root canal infections (Engström 1964). Furthermore, enterococci have the ability to survive in the root canal system as mono-infectants (Fabricius *et al.* 1982), and are hard to eliminate once present (Engström 1964).

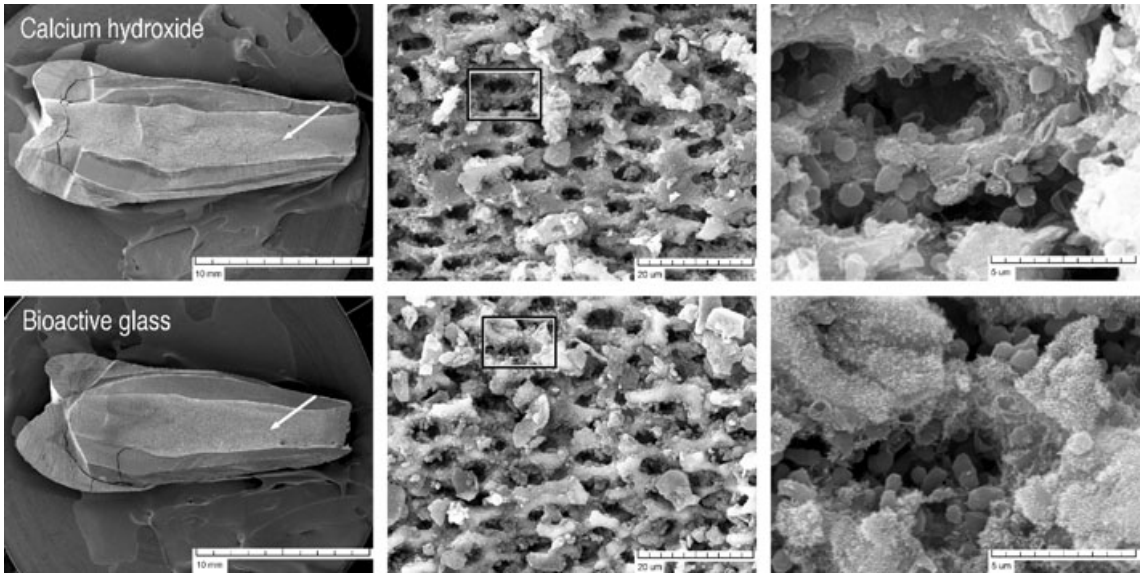

Figure 1 Longitudinally fractured contra-lateral premolars with open root apices as used for the microscopic part of this study. Centre panels: Details at 10 000× original magnification from the apical third of the root canal wall (left panels, arrows). Right panels: Details at 50 000× original magnification (from framed area in centre panels) depicting distinct differences between calcium hydroxide-dressed (top) and BAG-dressed (bottom) dentine wall.

In the present *ex vivo* model, the broth was changed according to the standard protocol used in bovine dentine blocks infected with *E. faecalis* (Haapasalo & Ørstavik 1987), resulting in predictably high numbers of viable enterococci. However, if the infection was left to develop for 2 weeks without changing the nutritional or metabolic output pool, a starved biofilm resembling the clinical situation more closely may have formed. This should be tested in future experiments.

Tooth crowns were flamed prior to sampling. Flaming is a standard laboratory procedure to eradicate microbiota from contaminated surfaces. The flaming procedure did not eliminate bacteria from the root canal, as shown in the positive control samples. Furthermore, no viable enterococci were recovered from swab samples off the flamed crown surface, as shown in preliminary experiments. The bacterial sampling was performed using files of increasing diameter to obtain consecutive dentine samples. Using this approach, however, curves in the canal system could not necessarily be negotiated, and dentine samples off the canal axis may have been harvested. Nevertheless, the current method gave some information on disinfection of dentinal tubules close to the main canal, as suggested by the fact that smaller files generally yielded less positive samples than counterparts of larger diameter. However, using such an approach, quantification of the bacteria made little sense, as it was impossible to standardize dentine yields. Consequently, an outcome dichotomized to 'growth' and 'no growth' was chosen for the microbiology part of this study.

Teeth were not irrigated with an antiseptic solution prior to dressing with the suspensions under investigation. The effects of the dressing materials proper could thus be studied. Relatively thin suspensions were rotated into the wet canal system. At least with calcium hydroxide, there is evidence that a thin suspension is more efficient in eliminating *E. faecalis* from root dentine than a thick mixture of calcium hydroxide powder with water (Behnen *et al.* 2001). No attempt was made to compact the materials into the canals, but rather multiple increments of the materials were rotated into the canal system to working length until the suspensions extruded back through the access cavity, suggesting a complete fill (Peters *et al.* 2005). While this approach appears to be ideal for calcium hydroxide, there is no data in the literature on how a BAG suspension should ideally be placed. Both materials used in the current investigation, i.e. the BAG and the calcium hydroxide powder, behave relatively similar when mixed to thin slurries. The BAG, however, takes much less water than calcium hydroxide to form a slurry of similar consistency. It is unknown at present

whether alternative placement methods and/or galenic compositions of BAG may improve the material's antibacterial effect in the root canal system.

The antimicrobial action of calcium hydroxide in suspension appears to be exclusively related to the pH generated in the material's environment (Proell 1949, Byström *et al.* 1985). Calcium hydroxide is poorly soluble in water. The powder in suspension is a depot for hydroxide ions, which are steadily released over time. The proteolytic and hyperosmotic pressure exerted by the hydroxide ions appears to disintegrate bacteria, as revealed by the SEM observations in the current study. The calcium hydroxide effect, however, is not only beneficial, as the proteolytic action may affect dentine stability via degradation of the collagen matrix (Grigoratos *et al.* 2001, Andreasen *et al.* 2002). The antibacterial BAG properties have also been related to pH, caused by the release of NaOH from the glass in aqueous environments (Allan *et al.* 2001, Zehnder *et al.* 2006). In addition, silica is released from the glass, a phenomenon that is increased in proximity to dentine (Zehnder *et al.* 2006). Silica can initiate Ca/P precipitations in the presence of excess Ca(II) and P(V) (Kangasniemi *et al.* 1993). As revealed by SEM analysis, apparent crystalline structures covered the inner root dentine surface exposed to the BAG suspension. It is highly unlikely that the surface characteristics of root canal dentine were changed by the fixation and later the washing process in buffer. Water-soluble compounds are at least partly washed off the dentine during that process; natural tooth components such as collagen and mineral, on the other hand, are not affected. Osmium tetroxide interacts primarily with lipids and, thus, stabilizes cell membranes (e.g. of bacteria). As a result, it avoids artefacts introduced by dehydration with organic solvents such as acetone, which dissolve the lipid component of the cell membranes. In summary, treatment with osmium tetroxide can be expected to preserve a closer to natural appearance of the microorganisms in the root canal. On the other hand, only minor interactions seem to occur between osmium tetroxide and collagen and, even less, dentine mineral. Therefore, the postfixation (with o.t.) is unlikely to alter the structural details of the walls of the root canals. Consequently, it would appear that at least part of the antimicrobial action of BAG is transmitted via calcification processes. This effect may hold some promise for future developments in dental materials, as calcification in the root canal system is a perfectly biocompatible process. However, the current BAG suspension was unable to rid the teeth of an *E. faecalis* infection, and material modifications appear to be necessary in the continued search towards a biocompatible antiseptic root canal dressing using glass materials.

Conclusions

Calcium hydroxide was an effective disinfectant in human teeth. In its current form, the BAG material appears to be an inferior root canal antiseptic in comparison with calcium hydroxide.

Acknowledgements

The authors thank Docent Eva Söderling for her advice and Jacqueline Hofmann for her help with the microscopy procedures.

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