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ABSTRACT

An ideal preparation of 45S5 bioactive glass suspensions/slurries for root canal disinfection should combine high pH induction with capacity for continuing release of alkaline species. The hypothesis of this study was that more material *per* volume of bioactive glass slurry is obtained with a micrometric material (< 5 µm particle size) or a micrometric/nanometric hybrid, rather than a solely nanometric counterpart. This should correlate with alkaline capacity and antimicrobial effectiveness. Slurries at the plastic limit were prepared with test and reference materials in physiological saline. Total mass and specific surface area of glass material *per* volume were determined. Continuous titration with hydrochloric acid was performed, and antimicrobial effectiveness was tested in extracted human premolars mono-infected with *E. faecalis* ATCC 29212 (N = 12 *per* material). While the nanometric slurry had a 12-fold higher specific surface area than the micrometric counterpart, the latter had a considerably higher alkaline capacity and disinfected significantly better (Fisher's exact test, P < 0.05). The hybrid slurry behaved similarly to the micrometric preparation.

KEY WORDS: bioactive glass, nanotechnology, Enterococci, root canal.

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Fine-tuning of Bioactive Glass for Root Canal Disinfection

INTRODUCTION

Bioactive glasses of the SiO₂-Na₂O-CaO-P₂O₅ type have recently been suggested as topical root canal disinfectants (Zehnder *et al.*, 2004). Similarly to calcium hydroxide, the most frequently advocated inter-appointment dressing, bioactive glasses disinfect their environment *via* the continuous release of alkaline species in a wet environment (Allan *et al.*, 2001). Calcium hydroxide and also bioactive glass suspensions are best administered as slurries that can be applied by means of a counter-angle handpiece and a lentulo spiral (Peters *et al.*, 2005). However, in contrast to calcium hydroxide, bioactive glasses do not weaken the dentin structure (Marending *et al.*, 2009). They release calcium, phosphate, sodium, and silica, and thus change slowly into pure inert calcium phosphate particles (Sepulveda *et al.*, 2002). Furthermore, bioactive glasses cause calcium phosphate precipitation in their environment (Kangasniemi *et al.*, 1993). Consequently, these materials transform from reactive local anti-septics into a bioactive hard-tissue-like structure over time.

Studies with a commercially available bioactive glass for dental application (S53P4, Abmindent, Abmin Technologies, Turku, Finland), however, have shown that such a material was inferior to calcium hydroxide as a disinfectant of human root canals mono-infected with *Enterococcus faecalis* (Zehnder *et al.*, 2006), a species with a high tolerance for alkaline biocides that has been associated with failed root canal treatments (Molander *et al.*, 1998). The glass used had an average particle size of 20 µm. Direct exposure tests with nanometric (< 100 nm) *vs.* micrometric (> 5 µm) bioactive glass particles in bacterial suspensions revealed that the antibacterial effect of bioactive glasses could be greatly enhanced by lowering their particle size and thus their immediate release of alkaline species (Waltimo *et al.*, 2007). In contrast, the antimicrobial effect is also related to the capacity of these materials to maintain an alkaline environment continuously over time or, in other words, their 'depot effect' (Gubler *et al.*, 2008). In the restricted volume of a root canal system, the depot effect might be related to the mass of bioactive glass material *per* total volume of the suspension/slurry.

In the current study, the following hypotheses were tested: (a) An aqueous slurry with nanometric bioactive glass powder contains substantially less material mass *per* volume than a counterpart of a micrometric glass; (b) because of this and the high reactivity of the nanometric material, the capacity for continuous release of alkaline species is lower in the nanometric compared with the micrometric glass slurry; and (c) a hybrid material containing both nanometric and micrometric material may combine both fast initial followed by continuous release of alkaline species, and might thus be ideal as a topical root canal disinfectant.

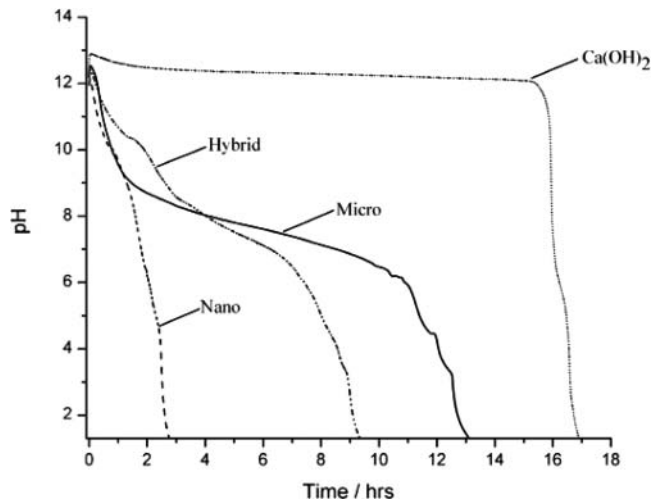


Figure. Continuous titration of the bioactive glass 45S5 and calcium hydroxide slurries ($N = 3$, five-fold dilutions, each) with 1 M HCl and a constant flow rate of 1.78 mL per hr.

MATERIALS & METHODS

Test and Reference Materials

Conventional bioactive glass 45S5 with a mean particle size of $< 5 \mu\text{m}$ (NovaMin Technology, US Biomaterials Corp., Alachua, FL, USA) was obtained from a commercial source. According to the manufacturer, this material consists of 45 wt% SiO_2 , 6% P_2O_5 , 24.5% CaO , and 24.5% Na_2O . Bioactive glass 45S5 of nanometric size was prepared by flame spray synthesis with suitable liquid metal carboxylate precursors as described previously (Brunner *et al.*, 2006). According to laser ablation inductively coupled plasma mass spectrometry, the composition of the nanometric bioactive glass was 44.7 wt% SiO_2 , 4.9 wt% P_2O_5 , 27.6 wt% CaO , and 22.8% Na_2O . A hybrid material, *i.e.*, a 50/50 wt% mixture of nanometric and micrometric bioactive glass, was ground with a mortar. Calcium hydroxide (Puriss) as a reference material was purchased from Riedel-deHaen Chemicals (Stoke Canon, Devon, UK).

The specific surface area of investigated materials was analyzed by nitrogen adsorption at 77 K with the Brunauer-Emmett-Teller (BET) method on a Tristar 3000 (Micrometrics, Norcross, GA, USA).

Capacity of Test and Control Slurries

We investigated the amount of material *per* volume of slurry by mixing the bioactive glass powders with unbuffered physiological saline (0.9 wt% NaCl), to achieve a maximal loading of the slurry at the plastic limit (the minimum amount of liquid needed to shape a powder into a paste-like slurry). Applicability of slurries into artificial root canals by means of a lentulo spiral was tested in plastic training blocks (Dentsply Maillefer, Ballaigues, Switzerland). Based on these values, a 10-mL suspension of each material was prepared and titrated so that alkaline capacity could be visualized. These suspensions were 5 times less concentrated than the above-mentioned slurries. Continuous titration with a flow rate of 1.78 mL per hr of hydrochloric acid (1 M) was performed with a peristaltic pump (ecoline VC-MS/CA8-6, Ismatec, Glattbrugg, Switzerland). The pH was determined by a calibrated pH electrode (Seven Easy, Mettler-Toledo, Greifensee, Switzerland). Rheological data of the applied slurries (original concentration) were measured on a MCR 300 (Physica, Anton-Paar, Ostfildern, Germany). In this apparatus, the slurries were sheared between 2 parallel plates at room temperature so that their viscosity could be measured.

Teeth

Teeth used for this investigation were premolars with fully formed apices extracted for orthodontic reasons. All teeth were free of caries and restorations. The current research protocol was according to the Guideline for Good Clinical Practice (ICH, Geneva, Switzerland) and did not alter the treatment plan of any of the involved individuals, who gave informed consent that their extracted teeth could be used for study purposes. The institutional ethics committee approved the procedures. In total, 60 premolar teeth were obtained from 22 individuals 12 to 32 yrs of age (median = 16 yrs). Teeth were externally cleaned and endodontically prepared with ProFiles (Dentsply Maillefer) 0.04 to size 45, followed by K-files up to size 60 at working length as described previously (Zehnder *et al.*, 2006). During instrumentation, root canals were irrigated with 10 mL of a 1% NaOCl solution administered *via* a 30-gauge irrigating needle (Hawe Neos, Bioggio, Switzerland). After instrumentation, all root canals were rinsed with 5 mL of 17% EDTA, followed by copious amounts of distilled water. In a modification of the previously published method (Zehnder *et al.*, 2006), the teeth were subsequently embedded in epoxy resin (Stycast, Emerson & Cuming, Westerlo, Belgium) cylinders for ease of handling and microbiological sampling. The coronal ends of the epoxy cylinders were ground back to the level of the tooth crown equator by means of a cast trimmer (Produits Dentaires S. A., Vevey, Switzerland). Finally, the coronal aspect of each cylinder was polished with polishing paper up to 1000 grit. Specimens were then sterilized overnight in ethylene oxide and kept in individual sterilization bags until further usage.

Infection

Teeth were divided into 4 similar experimental groups of 12 specimens each; 6 additional teeth served as negative and 6 as positive controls. All teeth were rinsed with 2 mL tryptic soy broth (TSB, Oxoid Ltd., Hampshire, England) *via* a 30-gauge needle (Hawe-Neos). Subsequently, teeth were placed in individual glass tubes containing 5 mL TSB. Test tubes containing the teeth were 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 *Enterococcus faecalis* ATCC 29212 in TSB spectrophotometrically adjusted to 10^7 cells/mL. Tubes were incubated in ambient air at 37°C for 3 wks. The broth was aseptically changed at two- to three-day intervals. Purity of the culture was checked after 1 and 3 wks of incubation by cultivation on tryptic soy agar (Oxoid) and subsequent observation of colony morphology as well as cellular characteristics after Gram staining. This and all the subsequent laboratory procedures were performed under aseptic conditions in a microbiological safety cabinet (SFE.120 EN, SKAN AG, Basel, Switzerland).

Test and Control Treatments

All teeth were rinsed with 2 mL of sterile saline solution. The 4 similar groups of experimental teeth were randomly allocated (www.random.org) to receive one of the following root canal dressings: calcium hydroxide, nanometric 45S5, micrometric 45S5, or the 50/50 45S5 hybrid material. Materials were mixed as described above and placed in the root canals by means of a lentulo spiral (Dentsply Maillefer) in a contra-angle handpiece (KaVo, Biberach, Germany). Access cavities were dried with compressed air, but canals were left wet prior to application of the topical antiseptics. Root canals were completely filled with the materials. Positive and negative control teeth were not medicated. All teeth were then transferred to individual sterile tubes containing glass beads soaked in 1 mL of saline. By closing the lids of the tubes,

we maintained a 100% humid environment. Specimens were incubated at 37°C for 10 days.

Harvesting of Dentin Samples

Before dentin chips were sampled, teeth were irrigated with 2 mL of sterile physiological saline. To prevent contamination from the outer tooth surfaces, we then briefly exposed the coronal surfaces of the specimens with the access cavities to a butane gas flame. To check sterility of the access, we swabbed the coronal surface of each specimen with a sterile cotton pellet, which was subsequently transferred to a tube containing TSB. Subsequently, dentin was cut in the apical area of the canal by an ISO-size 70 followed by an 80 and finally a 90 hand instrument (Dentsply Maillefer). The files were separated 5 mm from the tip by means of a sterile wire cutter. File tips were directly transferred to sterile tubes containing 5 mL TSB. In teeth with 2 canals, dentin chips were obtained from only 1 canal. Tubes containing the files or cotton pellets were incubated up to 5 days so that growth of *E. faecalis* could be detected. Purity of growth was assessed as described above.

Data Analysis

The numbers of specimens showing residual growth of *E. faecalis* per file size were compared between experimental groups (N = 12 each) by Fisher's exact test. Multiple testing was compared by Bonferroni's correction. The alpha-type error was set at 0.05.

RESULTS

The BET specific surface and total material surface area per volume of slurry varied between the different preparations (Table 1). The nanometric glass in the applied slurry had a substantially higher surface area than the micrometric material. In contrast, approximately five-fold less nanometric 45S5 total mass could be suspended in saline solution compared with the micrometric counterpart per volume of slurry at the plastic limit. The viscosity of the 4 applied slurries varied between 1 and 5 pascal seconds (Pa.s) (for comparison, glycerol has a viscosity of 1 Pa.s, honey between 2 and 10 Pa.s).

Titration of the five-fold-diluted slurries with 1 M HCl showed different buffer capacities for the bioactive glasses, while calcium hydroxide gave results expected for a strong-base/strong-acid titration. The nanoparticulate material had the weakest buffer effect, while the hybrid material had early-onset and long-term capacity, and the micron-sized particles had mainly a long-term effect (Fig.).

In the microbiological part of the study, all growth-positive samples showed pure cultures of *E. faecalis*. All the samples from the flamed access surfaces were growth-negative (Table 2), as were the dentin samples obtained from negative control specimens (N = 6, 3 samples per tooth). All 18 dentin samples from the

Table 1. Specifications of Bioactive Glass and Calcium Hydroxide Slurries at the Plastic Limit in Physiological Saline Used in the Current Study

Wt Composition of Suspended Material	Wt [µg] of Material Suspended per mm ³ of Slurry	BET* Specific Surface Area [m ² per g]	Total Material Surface Area [m ² per mm ³ of Slurry]
Nanometric 45S5 100%	170	71	0.012
Micrometric 45S5 100%	800	1.5	0.001
Nanometric 45S5 50%	600	28	0.017
Micrometric 45S5 50%			
Calcium hydroxide	600	15	0.009

* The specific surface area was measured by nitrogen adsorption at 77° K with the Brunauer-Emmett-Teller (BET) method.

Table 2. Residual Growth Ratio (sample-positive specimens/total specimens) in Samplings from Human Premolars Infected with *E. faecalis* ATCC 29212 after 10 Days of Test and Control Treatments

Root Canal Dressing	Access	Size 70	Size 80	Size 90
Nanometric 45S5 100%	0/12	11/12 ^{A*}	12/12 ^A	12/12 ^A
Micrometric 45S5 100%	0/12	1/12 ^B	1/12 ^B	1/12 ^B
Nanometric 45S5 50%				
Micrometric 45S5 50%	0/12	3/12 ^B	3/12 ^B	3/12 ^B
Calcium hydroxide	0/12	0/12 ^B	0/12 ^B	0/12 ^B

* Identical superscript letters indicate that there was no significant difference between respective treatments within a sampling level at the 0.05 level (Fisher's exact test, Bonferroni).

positive control teeth (N = 6) showed vigorous growth. Residual growth was observed in all teeth dressed with a nanometric 45S5 slurry. This was in contrast to teeth dressed with the micrometric material, the hybrid between nanometric and micrometric glass, and calcium hydroxide, which were similarly effective (Table 2).

DISCUSSION

The current study showed that not only the specific surface area, but also the total mass of material per volume in bioactive glass slurries is important when used as topical root canal disinfectants. As shown here, this limits the usefulness of bioactive glass nanoparticles alone for the current purpose, since relatively little bulk material can be administered into a clinically applicable slurry. This results in high initial reactivity, but little capacity of the slurry to maintain an alkaline environment over time. Analysis of the present data demonstrates that, while calcium and silica species in an alkaline environment also play a role (Gubler *et al.*, 2008), alkaline capacity of a bioactive glass preparation is the main feature that mediates its antimicrobial effect in the root canal system.

The model with human teeth mono-infected with the *E. faecalis* type strain used in this study yielded repeatable results comparable with those obtained in clinical studies (Ørstavik *et al.*, 1991). Depending on the type of infection, however, clinical cases may yield different results. The main goal of the present study was to assess weigh the effect of total mass per volume of a bioactive glass slurry against its specific surface area under standardized conditions. Furthermore, the sequential sampling with files of increasing size cannot predictably prevent microbial

carry-over. Nevertheless, disinfection of the dentin close to the pulp with persisting infection in deeper layers (*i.e.*, those sampled with larger files) has recently been reported when teeth in the current model were disinfected with a bioactive glass of large particle size (Zehnder *et al.*, 2006).

The results reported here are seemingly in contrast to those of a recently published study in which bioactive glass 45S5 was more efficient in its nanometric compared with its micrometric form (Waltimo *et al.*, 2007). However, in that study, thin (1:10, wt/vol) slurries were used and tested in direct exposure tests to clinical strains of *E. faecalis*. In that set-up, excess glass material was in the system, and consequently, reactive surface area *per* volume was the main feature responsible for the antimicrobial effect. However, in the root canal system, space is limited for obvious reasons and consequently, alkaline biocides are best administered as aqueous slurries close to their plastic limit to permit optimal placement and allow for maximal long-term dissolution of alkaline species into the aqueous phase. This was corroborated by the current investigation.

The high effectiveness of the micrometric glass used in the current study was somewhat surprising, since a similar material showed less effect in a previous study under similar conditions (Zehnder *et al.*, 2006). However, the glass used in the current study had a relatively small particle size (average, < 5 μm) compared with that in the previous study (average, 20 μm), and thus triggered higher pH levels. Nevertheless, the micrometric material *per se* may still not be ideal as a root canal dressing, since it may still allow for recontamination of the root canal system with sub-micrometric microbiota (Zehnder *et al.*, 2007).

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