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**ORIGINAL ARTICLE**

# Bone powder enhances the effectiveness of bioactive glass S53P4 against strains of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in suspension

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**Abstract**

***Objective***. To assess whether bone powder in suspension enhances the antimicrobial efficacy of bioactive glass S53P4 against Gram-negative microbiota commonly associated with peri-implant disease. ***Methods***. Standardized suspensions of *Porphyromonas gingivalis* ATCC 33277 and YH 3, as well as *Actinobacillus actinomycetemcomitans* ATCC 29523 and KK 2 were added to 24-h suspensions of bioactive glass S53P4 with ground bovine bone powder, decalcified bone, or hydroxylapatite powder. Recovery of viable bacteria was assessed using anaerobic culture methods. As a reference, the antibacterial effect of an inert borosilicate powder with a particle size corresponding to that of the bioactive glass was tested. Counts of bacteria suspended in a pure unbuffered saline solution served as controls. ***Results***. A significant drop in viable microorganisms was observed in suspensions of bioactive glass and bone powder compared to counterparts of pure bioactive glass. In contrast, neither the presence of hydroxylapatite powder nor the presence of decalcified bone in suspension caused any increase in bioactive glass killing efficacy on the microorganisms under investigation. Inert borosilicate glass showed no antibacterial effects *per se* or in combination with bone powder. ***Conclusion***. The antimicrobial effect of a combined bioactive glass-ground bone powder suspension was an *in vitro* observation which should be confirmed using adequate *in vivo* models.

**Key Words:** *Actinobacillus actinomycetemcomitans, bioactive glass, bone, peri-implantitis, Porphyromonas gingivalis*

## Introduction

Bioactive glasses of the $SiO_2$-$Na_2O$-CaO-$P_2O_5$ system show antimicrobial properties against oral pathogens *in vitro* [1]. Interestingly, dental hard tissues in a liquid environment appear to enhance this effect against Gram-positive facultative microorganisms [2]. Dentin powder in suspension triggered the ionic dissolution of bioactive glass S53P4, leading to increased local pH and silica levels, which interfered with bacterial viability [3]. Hydroxylapatite or demineralized organic dentin components were unable to cause the effect observed with whole dentin, suggesting that the complex organic–inorganic dentin surface would act as a recipient for ions in solution and thus as a catalyst for the bioactive glass dissolution [3].

It was the purpose of the present *in vitro* study to assess whether bone, with its composition comparable to dentin [4], would enhance the antimicrobial efficacy of bioactive glass S53P4 against Gram-negative microbiota commonly associated with peri-implant disease [5]. Hypothetically, it was assumed that ground bone powder in suspension would boost the antimicrobial bioactive glass effect.

## Material and methods

The bioactive glass powder used in the current study was S53P4 (AbminDent 1, Vivoxid, Turku, Finland). It is composed of 53% $SiO_2$ (wt/wt), 23% $Na_2O$, 20% CaO, and 4% $P_2O_5$, and was prepared from reagent grade $Na_2CO_3$, $CaHPO_4 \cdot 2H_2O$, $CaCO_3$ (Merck, Darmstadt, Germany), and Belgian

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sand, as described elsewhere [1]. The particle size of the S53P4 was $\leq$45 μm, with an average of approximately 20 μm. Inert borosilicate powder was used as a reference material (Microglass Glass Flakes RCF 015, Mühlheimer GmbH, Bärnau, Germany). The borosilicate powder had an average particle size of 19 μm, which was similar to that of the bioactive glass used in the current study. Particle size was determined in aqueous suspensions using laser diffraction analysis (CILAS 1064, Marcoussis, France). Bone powder was prepared from a bovine mandibular jaw obtained from the local abattoir freshly after slaughtering. The jaw was mechanically cleaned from all soft tissue, cut to pieces, and freeze-dried in a desiccator. Cortical and spongious bone was then ground in a ball grinder (MM 200, Retsch, Haan, Germany) for 2 min. Decalcification of bone was performed by suspending 1-gram aliquots of the bone powder in 100 ml of 17% EDTA solution at pH 8 under constant stirring at room temperature for 3 days with daily changes of EDTA. This procedure rid the bone from >99.9% of detectable calcium, as assessed using atomic absorption spectrophotometry (Model 2380; Perkin-Elmer, Norwalk, Ct., USA). The washed decalcified bone was finally suspended in saline, and aliquots of the suspension were stored frozen before use in the experiments. Hydroxylapatite powder (BDH Chemicals, Poole, England) was used as a further reference material. Bone powder, bioactive glass, and all the reference particles were sterilized in an ethylene oxide sterilizer (Sterivac 4XL; 3M, St. Paul, MN, USA).

*Porphyromonas gingivalis* ATCC 33277 and a clinical isolate YH 3 were pre-cultivated anaerobically (80% $N_2$, 10% $CO_2$, 10% $H_2$) on Brucella agar plates (Difco Laboratories, Detroit, Mich., USA). The cells were harvested after 6 days' growth, washed once in 0.9% NaCl (saline), and finally adjusted in saline to an optical density of approximately 1.0 ($A_{700}$) corresponding to $10^7$–$10^8$ colony forming units (CFU)/ml. *Actinobacillus actinomycetemcomitans* ATCC 29523 and a clinical isolate KK 2 were pre-cultivated in BHI (Brain Heart Infusion, Unipath Ltd, Hampshire, England). After 18 h of growth, the cells were harvested by centrifugation (10 000 $\times g$ for 10 min), washed in saline and spectrophotometrically adjusted to an optical density of approximately 1.0 in saline ($10^7$–$10^8$ CFU/ml). All saline solutions used in the present study were sterile, of physiologic concentration (0.9% wt/vol) and unbuffered. Before use, they were anaerobically pre-incubated overnight.

In sterile Eppendorf tubes, 30 mg bioactive glass, or 22 mg bone powder+30 mg bioactive glass were suspended in 100 μl of saline and pre-incubated at 37°C for 24 h. Similar suspensions of bone powder *per se* (22 mg/100 μl), decalcified bone (amount corresponding to 22 mg non-decalcified bone/100 μl) *per se* or plus bioactive glass, hydroxylapatite powder (22 mg/100 μl) plus bioactive glass, or bone powder plus inert borosilicate glass (30 mg/100 μl) as well as borosilicate *per se* were prepared in saline and incubated at 37°C for 24 h. The saline solution proper served as the control in all experiments. Pre-incubated suspensions of the test and reference materials or pure saline controls were thoroughly mixed with 100 μl of the standardized bacterial suspensions. The mixtures were incubated at 37°C. Owing to rapid loss of viability, the incubation time for *P. gingivalis* was 10 min, but for *A. actinomycetemcomitans* incubation times up to 30 min were used. To stop the reactions, 800 μl of saline was added, the suspensions were vortexed, and the materials were allowed to settle for 2 min. In pilot experiments, mild sonication was used in addition to vortexing, as described earlier [3]. Viability of the cells in solution was assessed using serial dilutions of the supernatants by plate culturing anaerobically on Brucella agar (*P. gingivalis*; 6 days) or blood agar (*A. actinomycetemcomitans*; 3 days) at 37°C. The blood agar was obtained from Orion Diagnostica, Espoo, Finland.

All experiments were repeated at least twice. Results are expressed as means of $\log_{10}$ CFU $\pm$ standard deviations (SD).

## Results

Pre-incubation of bioactive glass with bone powder for 24 h resulted in an anti-bacterial suspension, which was significantly more efficient than a corresponding pure bioactive glass suspension. The boosting effect was observed with both *P. gingivalis* and *A. actinomycetemcomitans*. In pilot experiments, a mild 10-s sonication of the suspensions prior to cultivation did not increase the bacterial yield, while this sonication *per se* did not affect viability of the microorganisms under investigation. In these experiments, neither bacterial aggregates nor adherent cells were observed microscopically.

For *P. gingivalis*, short incubation times had to be used because of rapid loss of viability under the experimental conditions; the decrease in viability being approximately 0.5–1.0 $\log_{10}$ CFU during the 10-min incubation. Thus, the linearity of the reaction could not be tested. For the results obtained for the *P. gingivalis* type strain ATCC 33277, see Figure 1. The bioactive glass plus bone suspension had an antimicrobial effect superior to bioactive glass alone, while the reference materials bone and inert glass with/without bone showed no effect on cell viability (Figure 1). Decalcified bone and hydroxylapatite did not boost the effect of the bioactive glass (not shown). The results for the clinical isolate were in line with corresponding observations obtained with the type strain. A reduction of mean $\log_{10}$ CFU of *P. gingivalis* YH 3 after 10

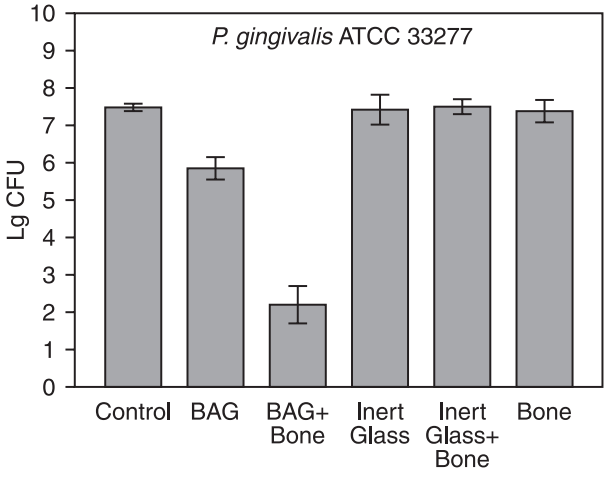


Figure 1. Survival of planktonic *P. gingivalis* ATCC 33277 ($\log_{10}$ CFU $\pm$SD) incubated for 10 min in test and reference suspensions or an unbuffered saline solution (Control). BAG = bioactive glass S53P4.

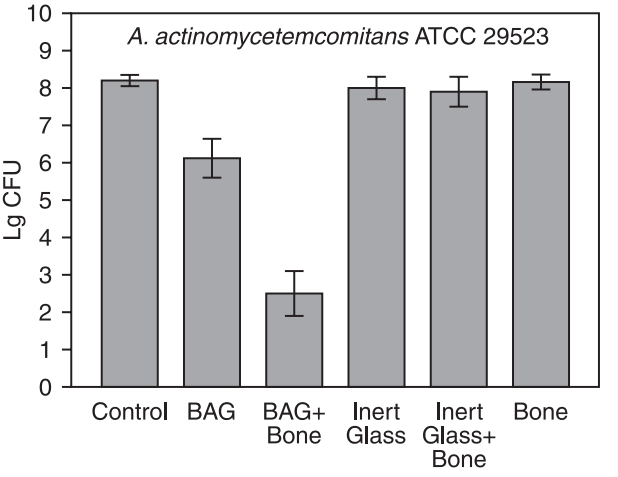


Figure 3. Survival of planktonic *A. actinomycetemcomitans* ATCC 29523 ($\log_{10}$ CFU $\pm$SD) incubated for 30 min in test and reference suspensions or an unbuffered saline solution (Control). BAG = bioactive glass S53P4.

min of incubation in the bioactive glass suspension was 1.7, compared to a corresponding reduction in the combined bioactive glass and bone suspension of 4.0.

For *A. actinomycetemcomitans*, longer incubation times could be used; both the antimicrobial effect of bioactive glass and the boosting effect of the bone increased with time when incubation times of 15 and 30 min were tested (Figure 2). With the type strain *A. actinomycetemcomitans* ATCC 29523, there was a decrease in viable bacteria of more than five orders of magnitude after 30 min (Figure 3). As observed with the *P. gingivalis* type strain, the reference materials bone and inert glass with/without bone (Figure 3) had no effect on *A. actinomycetemcomitans* ATCC 29523 recovery. Again, decalcified bone and hydroxylapatite did not boost the effect of bioactive glass (not shown). With the clinical isolate KK 2, the mean recovery of viable bacteria was $6.8 \pm 0.1$ $\log_{10}$ CFU after 30 min of incubation in saline. From the bioactive glass suspension, $4.2 \pm 0.4$ $\log_{10}$ CFU was recovered. No viable bacteria were detected in supernatants of combined bioactive glass and bone powder suspensions.

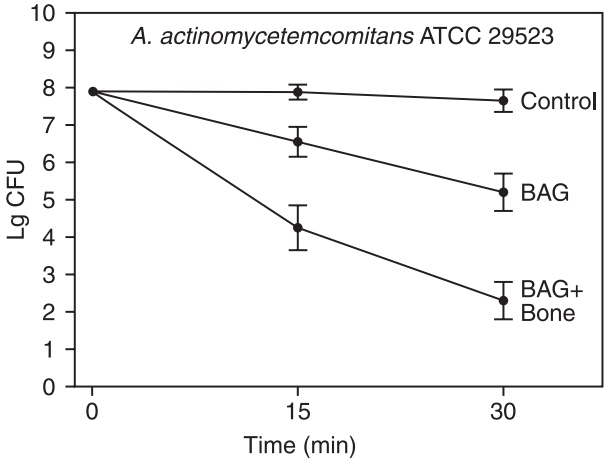


Figure 2. Time effect of bioactive glass S53P4 (BAG) and bioactive glass plus bone powder suspensions on recovery of *A. actinomycetemcomitans* ATCC 29523 ($\log_{10}$ CFU $\pm$SD). Standardized suspensions of the bacterium were added to suspensions of the materials pre-incubated for 24 h.

## Discussion

The effect of bone on the antimicrobial efficacy of bioactive glass S53P4 suspensions against *P. gingivalis* and *A. actinomycetemcomitans* was tested in the current study. Referring to previous observations using dentin [2,3], it was assumed that bone, with its comparable composition, could also boost the antimicrobial efficacy of bioactive glass. In a previous investigation on the effect of dentin powder on the dissolution of bioactive glass in suspension [3], *Enterococcus faecalis* was used as the test organism, since this species has been commonly associated with persistent root canal infections [6]. In the current study, microorganisms more relevant for periodontal and peri-implant diseases were chosen. In the treatment of these pathoses, a therapeutic or preventive use of bone substitute materials could be advantageous [7].

Bioactive glass powder in the form of an aqueous paste has broad antimicrobial activity against oral pathogens including *A. actinomycetemcomitans* [1]. The high solubility of the bioactive glass surface results in low adhesion of bacteria to the material and poor biofilm formation on it [1,8]. The antimicrobial activity of aqueous suspensions of bioactive glasses of the $SiO_2$-$Na_2O$-CaO-$P_2O_5$ system is largely attributed to their high pH and non-physiological concentrations of ions in their environment [1,3,8]. As shown in earlier experiments, the addition of dentin powder to bioactive glass S53P4 suspensions increased the dissolution of the glass, resulting in increased pH, osmolarity, and

silica concentrations [3]. Silica has been suggested to boost the antimicrobial effect of a high-pH environment against *Escherichia coli* [3]. Bone powder with a similar composition to dentin [4] should also increase the dissolution of bioactive glass. In fact, we found earlier that bone powder boosted the antimicrobial efficacy of bioactive glass S53P4 against *Enterococcus faecalis* ATCC 29212 to a similar extent as dentin (Zehnder, unpublished results). Hypothetically, the complex surface of mineralized collagen matrix may act as catalyst for the dissolution of bioactive glass S53P4 components in aqueous suspensions. Similar to previous observations with dentin, no antimicrobial boosting effect was observed when decalcified bone or hydroxylapatite was used instead of whole bone powder. In conclusion, the presence of dentin and bone powder in suspension appears to exert similar effects on bioactive glass S53P4. It must be cautioned, however, that all the current results were obtained in a non-buffered environment, and hence further studies on the boosting effect of bone on the antimicrobial efficacy of bioactive glass in simulated body fluids are necessary.

Evaluation of possible clinical relevance of the current findings remains for further investigations. In treatment of peri-implant disease, débridement of the exposed implant surface followed by subsequent filling of the defect are essential for re-osseointegration [10]. Autogenous bone, if available, appears to be the best single filling material [11]. Artificial materials such as bioactive glass particles have also been used to fill bony defects around implants. The results with bioactive glass, however, have not been encouraging [12]. Combining a bioactive glass material with autogenous bone particles for bone regeneration is a concept that has received little attention as of yet, despite the fact that promising results have been reported [13]. As peri-implant disease is induced and/or supported by microbiota, the antimicrobial properties of such a combination may be advantageous.

In conclusion, under the conditions of the present study, ground bone powder increased the antimicrobial efficacy of bioactive glass S53P4 in aqueous suspension. This finding may open new strategies in the treatment of peri-implant disease. The antimicrobial effect of a combined bioactive glass-ground bone powder suspension was an *in vitro* observation which should be confirmed using adequate *in vivo* models.

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