Biofilm Removal and Antimicrobial Activity of Two Different Air-Polishing Powders: An In Vitro Study

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Background: Biofilm removal plays a central role in the prevention of periodontal and peri-implant diseases associated with microbial infections. Plaque debridement may be accomplished by air polishing using abrasive powders. In this study, a new formulation consisting of erythritol and chlorhexidine is compared with the standard glycine powder used in air-polishing devices. Their in vitro antimicrobial and antibiofilm effects on *Staphylococcus aureus, Bacteroides fragilis*, and *Candida albicans* are investigated.

Methods: Biofilm was allowed to grow on sandblasted titanium disks and air polished with glycine or erythritol-chlorhexidine powders. A semiquantitative analysis of biofilm by spectrophotometric assay was performed. A qualitative analysis was also carried out by confocal laser scanning microscopy. Minimum inhibitory concentrations and minimum microbicidal concentrations were evaluated, together with the microbial recovery from the residual biofilm after air-polishing treatment.

Results: The combination of erythritol and chlorhexidine displayed stronger antimicrobial and antibiofilm activity than glycine against all microbial strains tested.

Conclusion: Air polishing with erythritol-chlorhexidine seems to be a viable alternative to the traditional glycine treatment for biofilm removal. *J Periodontol 2014;85:e363-e369.*

KEY WORDS

Anti-infective agents; biofilms; dental polishing; erythritol; peri-implantitis; periodontitis.

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Subgingival biofilm removal is a fundamental part of periodontal therapy. Microbial biofilms are populations of microorganisms that are clustered at an interface (mostly solid–liquid) and usually enclosed within an extracellular polymeric matrix.[1] Periodontitis and peri-implantitis are bacterial infections associated with a complex microbiota of the dental biofilm that induce a local and systemic inflammatory response, leading to periodontal or peri-implant tissue breakdown.[2] Hand instruments and sonic or ultrasonic scalers may be used for debridement, though their use can be challenging and time consuming and may cause root damage over time.[3-6] Biofilm removal may also be achieved through the use of air-polishing devices.[7-10] However, conventional air polishing by means of water and sodium bicarbonate can be extremely abrasive to root cementum and dentin.[11-13] A less aggressive method using the amino acid glycine has been shown to induce minimum tooth and implant surface alterations while still removing biofilm with efficacy in vitro and in vivo.[14-18] In addition, this method has been used in the treatment of peri-implantitis and has been shown to be safe and provide good clinical results.[19,20]

More recently, erythritol was also adopted for use in air-polishing devices. Erythritol is a natural sugar alcohol produced by the reduction of erythrose.[21] It is

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efficiently excreted by the kidneys, so it has less capability to cause changes in glucose and insulin plasmatic levels or induce gastrointestinal side effects than other sugar alcohols.[22-24] Recently, antibiofilm activity of erythritol has been pointed out.[25,26] Hashino et al. showed that erythritol altered the microstructure and metabolomic profile of the biofilm produced by *Streptococcus gordonii* and *Porphyromonas gingivalis* under in vitro conditions.[25] Moreover, a randomized controlled trial demonstrated that erythritol air polishing was effective in removing dental plaque during repeated instrumentation of residual pockets in supportive periodontal treatment.[26]

Chlorhexidine is a broad-spectrum antimicrobial agent used as an ingredient of mouthwashes or as an irrigant for periodontal pockets and infected root canals. Chlorhexidine is effective in reducing the viability of biofilm-forming microorganisms, although it is unable to disrupt polymicrobial biofilms in in vitro and in situ studies.[27-34] Recently, a new formulation consisting of a combination of erythritol and chlorhexidine has been designed for biofilm removal. In the present study, the new formulation is compared with the standard glycine powder used in air-polishing devices. In particular, to show the effects of the new formulation versus the standard one, this study assesses: 1) the in vitro antimicrobial effect of the materials against oral microorganisms; 2) their ability to reduce the viable cell number; and 3) their ability to mechanically reduce the biofilm.

MATERIALS AND METHODS

Microbial Strains

Three types of biofilm-producing microorganisms found in several oral diseases were chosen for this study. In particular, an aerobic Gram-positive strain (*Staphylococcus aureus*), an anaerobic Gram-negative strain (*Bacteroides fragilis*), and a yeast strain (*Candida albicans*) were used. All strains have been previously selected for their ability to produce biofilm. *S. aureus* and *C. albicans* have been identified among species characteristic of peri-implantitis but not periodontitis,[35-38] whereas *B. fragilis* is an opportunistic periodontopathogen often present in various chronic endodontic and periodontal infections as well as in dental caries.[39-42]

Glycine and Erythritol Powders

Two different formulations are compared in this study: a powder (granulometry 25 μm) consisting of glycine and amorphous silica‖ and a powder (granulometry 14 μm) consisting of erythritol (99.7%), chlorhexidine (0.3%), and amorphous silica.¶

Determination of the Minimum Inhibitory Concentration and Minimum Microbicidal Concentration

The minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial substance that inhibits the visible growth of a microorganism, was determined by broth microdilution method. Briefly, a suspension in brain-heart infusion broth# was prepared for each strain, with an optical density equal to 0.5 McFarland standards (1×10^8 colony-forming units [CFU]/mL). After obtaining a concentration of 1×10^4 CFU/mL using appropriate dilutions, 20 μL of each suspension was inoculated in a 96-well microplate containing 180 μL of a serial two-fold dilution of the material under evaluation. Positive controls were performed by inoculating the microbial suspension in brain-heart infusion broth alone. MIC values were read visually after 24 hours of incubation at 37°C in proper conditions. The assay was conducted in duplicate for each strain. A minimum microbicidal concentration (MMC) test was also carried out. The MMC, defined as the lowest dilution of an antimicrobial substance that kills a microorganism, was determined by subculturing 10 μL microbial suspension from MIC tests. MMC values were read after incubation for 24 hours at 37°C in proper conditions.

Biofilm Development

To reproduce in vivo conditions, biofilm was allowed to grow on substrates mimicking those used in dentistry. In particular, tests were performed on disks made of titanium. Sterilized sandblasted titanium disks with a diameter of 25 mm and a thickness of 5 mm** were used. Strains were grown on titanium disks in six-well plates containing tryptic soy broth†† for 48 hours at 37°C. All microorganisms were grown in tryptic soy broth except for *B. fragilis*, which needed a supplement of 5% blood to the growth medium. Then, growth medium was removed, together with non-adherent microorganisms, and new broth was added. Plates were incubated at 37°C in proper conditions for an additional 24 hours, until a visible biofilm was obtained. Six titanium disks were prepared for each of the three strains, for a total of 18 disks. Within the six disks, two were air polished with a jet of water alone (mechanical control), two with glycine, and two with erythritol/chlorhexidine.

Air Polishing

Treatment was performed by means of an air-polishing device.‡‡ Briefly, after growth medium removal, disks were rinsed three times with sterile saline solution to remove non-adherent microorganisms. Then disks were air polished with glycine or erythritol-chlorhexidine. The angle of the handpiece tip to the disk surface was 30 to 60°. The pressure of the

‖ E.M.S. Electro Medical Systems, Nyon, Switzerland.
¶ E.M.S. Electro Medical Systems.
# bioMérieux, Marcy-l'Etoile, France.
** Adler Ortho, Cormano, Italy.
†† bioMérieux.
‡‡ E.M.S. Electro Medical Systems.

powder-water jet was the minimum allowed by the instrument. The handpiece was guided in a circular motion at a working distance of 20 mm. The instrumentation time was limited to 5 seconds, as recommended by the manufacturer. Mechanical controls were air polished under the same conditions with a flux of water only.

Microbial Recovery Into the Residual Biofilm

To investigate the effects of the three treatments on the viability of microbial cells embedded into the residual biofilm, the disks prepared and treated as described above were placed into sterile containers with 10 mL saline solution and sonicated for 5 minutes at room temperature in an ultrasonic bath[§§] using a frequency of 30 KHz and power of 300 W. Serial 10-fold dilutions in saline solution were prepared for each sample and plated on solid growth medium.[‖‖] After incubation at 37° for 16 to 18 hours, the number of cells was calculated as CFU per milliliter. The experiments were conducted in duplicate, and the results are expressed as mean ± SD.

Semiquantitative Analysis of Biofilm by Spectrophotometric Assay

The amount of biofilm was determined by a spectrophotometric assay, adopting the method developed by Christensen et al.[43] Tests were performed on titanium disks treated by air polishing, as described above. After treatment, disks were air dried and stained by immersion in a solution of 5% crystal violet. The dye in excess was removed by washing disks with saline, and then disks were completely air dried. The air-dried disks were placed in six-well plates containing 3 mL of 96% ethanol to resolubilize the dye included in the biofilm. Crystal violet absorbance was measured at 595 nm using a spectrophotometer.

Confocal Laser Scanning Microscopy Analyses

For confocal laser scanning microscopy (CLSM), *S. aureus* microbial biofilm was grown on titanium disks and treated as described above. Biofilm was stained with a fluorescent stain[¶¶] containing a mixture of two dyes: green fluorescent nucleic acid stain[##] (green stain) and propidium iodide (red stain). The green stain generally labels all bacteria in a population, whereas propidium penetrates only bacteria with damaged membranes. Thus, bacteria with intact cell membranes (i.e., live) stain fluorescent green, whereas bacteria with damaged membranes (i.e., dead) stain fluorescent red. Disks were incubated with fluorescent stains at room temperature for 15 minutes in the dark. A coverslip was placed on the specimen, and the stained biofilm was examined with a confocal microscope*** using a 20× dry objective plus a 2× electronic zoom. A 488-nm laser line was used to excite the green fluorescent stain while the fluorescence emission was detected at 500 to 540 nm. Propidium iodide was excited with a 561-nm laser line while its fluorescence emission was detected at 600 to 695 nm. Using a third laser line (633 nm) in a reflection mode, it was possible to determine both titanium disk (starting acquisition point) and cover slip (ending acquisition point) reflecting surfaces. Images from five randomly selected positions were acquired for each disk. For each of them, sequential optical sections of 2 μm were collected along the *z*-axis over the complete thickness of the sample. The resulting stacks of images were analyzed and subsequently rendered into three-dimensional mode using image analysis software.[†††]

Statistical Analyses

Results are expressed as mean ± SD. Comparisons between groups were done using unpaired Student *t* test. A probability value of $P = 0.05$ was used as the significance level.

RESULTS

Determination of MIC and MMC

Results are shown in Table 1. MIC and MMC are expressed as dilutions from the initial solution produced by the instrument. Both glycine and erythritol-chlorhexidine displayed inhibitory activity against the tested strains of *S. aureus, B. fragilis*, and *C. albicans*. The erythritol-chlorhexidine powder was more effective than glycine. Significant differences (>2 dilutions) were found between the MIC values of erythritol and glycine: MIC values of erythritol-chlorhexidine ranged from 1:64 to 1:512, and MIC values of glycine ranged from 1:8 to 1:16. Moreover, glycine did not show a microbicidal effect. In fact, MMC values could not be determined at the concentrations tested. In contrast, erythritol-chlorhexidine demonstrated a microbicidal effect against all the tested microorganisms.

Microbial Recovery Into the Residual Biofilm

The number of cells recovered into the residual biofilm after mechanical (control), glycine, or erythritol-chlorhexidine treatments are reported in Table 2. Compared with the mechanical treatment only, both glycine and erythritol-chlorhexidine treatment induced a reduction in the number of surviving cells for all microbial strains tested. Glycine was less effective against *S. aureus* than against *B. fragilis* and *C. albicans* strains. In fact, the reduction of surviving

§§ VWR, Milan, Italy.
‖‖ Adler Ortho.
¶¶ Filmtracer LIVE/DEAD Biofilm Viability Kit, Molecular Probes, Thermo Fisher Scientific, Waltham, MA.
SYTO 9, Molecular Probes, Thermo Fisher Scientific.
*** Leica model TCS SP5, Leica Microsystems, Mannheim, Germany.
††† Volocity, Perkin Elmer, Waltham, MA.

Table 1.

Determination of MIC and MMC (dilutions from the initial solution produced by the instrument)

Microbial Strain	MIC		MMC	
	Glycine	Erythritol-Chlorhexidine	Glycine	Erythritol-Chlorhexidine
S. aureus	1:8	1:512	>1:2	1:16
B. fragilis	1:16	1:256	>1:2	1:128
C. albicans	1:8	1:64	>1:2	1:32

Table 2.

Microbial Recovery Into the Residual Biofilm (CFU × 10^7/mL)

Microbial Strain	Control	Glycine	Erythritol-Chlorhexidine
S. aureus	7.2 ± 0.4	6.1 ± 0.3	3.8 ± 0.4*
B. fragilis	14 ± 2.8	9.7 ± 0.3	7.1 ± 0.4*
C. albicans	2.1 ± 0.2	1.4 ± 0.7	0.9 ± 0.1*

* $P < 0.05$.

cells after glycine treatment was ≈30% for *B. fragilis* and *C. albicans*, but only 15% for *S. aureus*. In contrast, erythritol-chlorhexidine caused a reduction of ≈50% in the number of surviving cells for all the tested microbial strains ($P < 0.05$).

Semiquantitative Analysis of Biofilm by Spectrophotometric Assay

Results of semiquantitative analysis of biofilm are shown in Figure 1. Erythritol-chlorhexidine air polishing was effective in reducing the biofilm produced by all microorganisms tested. The difference between absorbances of control and treated samples (erythritol) was significant for all strains ($P < 0.001$). Glycine air polishing was effective against *C. albicans* and *B. fragilis* ($P < 0.001$) but not against *S. aureus* biofilm. Moreover, erythritol-chlorhexidine was more effective than glycine ($P < 0.001$). The activity was significant for all strains, including *S. aureus*.

CLSM Analyses

S. aureus, C. albicans, and *B. fragilis* biofilm was reduced by erythritol-chlorhexidine air polishing. The reduction in the fluorescence of biofilm due to erythritol-chlorhexidine treatment was more evident compared with glycine, as shown in images of *S. aureus* biofilm obtained with CLSM (Fig. 2).

DISCUSSION

Air polishing is used in several dentistry applications that require the removal of bacterial biofilm. Among them, the management of periodontitis and peri-implantitis is of considerable importance, as these diseases represent a worldwide public health problem. It is known that the prevalence of both periodontitis and peri-implantitis is very high in the population. Periodontitis is considered to be the second most common chronic disease worldwide after dental decay, with a prevalence of 30% to 50% in the United States. In 2010, chronic periodontitis affected ≈10.8% of the world population.[44]

Moreover, data suggest that the prevalence of peri-implantitis is in the range of 16% to 25% of patients.[45] Various studies have highlighted a cause-and-effect relationship between microbial plaque colonization and the pathogenesis of periodontal and peri-implant infections.[46-48] It was then assumed that the removal of microbial plaque biofilm would be a prerequisite for the management of both periodontitis and peri-implantitis. Several treatment procedures have been recommended, such as mechanical and ultrasonic debridement or laser application. An air abrasive method for removing bacterial biofilm from tooth surfaces has also been used.[7,11] This procedure has been adopted in the treatment of peri-implant infections, showing no serious adverse effects.[49] However, in vitro data showed that the use of highly abrasive substances (e.g., sodium bicarbonate) might cause alterations in teeth and implant surface characteristics. A less aggressive method using the amino acid glycine has been shown to efficiently remove bacterial plaque. Moreover, compared with previous methods, the reduced pressure and the shorter treatment time (5 seconds per site) have been shown to cause less trauma to biologic tissues and implant surfaces and to be more comfortable for patients.[18] Recently, the sugar alcohol erythritol has also been introduced in air-polishing technology. A few studies provide evidence of its efficacy in removing biofilm in vivo and in vitro.[25,26] Combining agents able to disrupt the structural integrity of biofilm (e.g., erythritol) with chemical agents with established antimicrobial properties (e.g., chlorhexidine) might represent a new strategy for removing biofilm.

The present study is designed to evaluate the effect of glycine and a combination of erythritol and chlorhexidine on the biofilm produced by *S. aureus, B. fragilis*, and *C. albicans* in vitro. MIC and MMC of glycine and erythritol-chlorhexidine powders were also evaluated, together with the microbial recovery into the residual biofilm after air-polishing treatment. Both treatments showed inhibitory activity, as

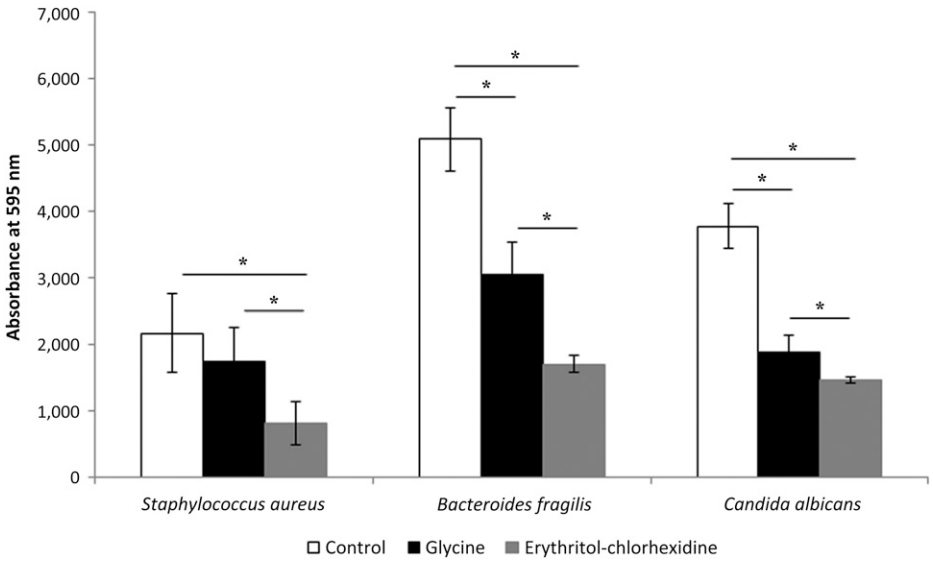

Figure 1.
Semiquantitative analysis of biofilm by spectrophotometric assay. Data are expressed as mean absorbance. Error bars represent standard deviation. *P <0.001.

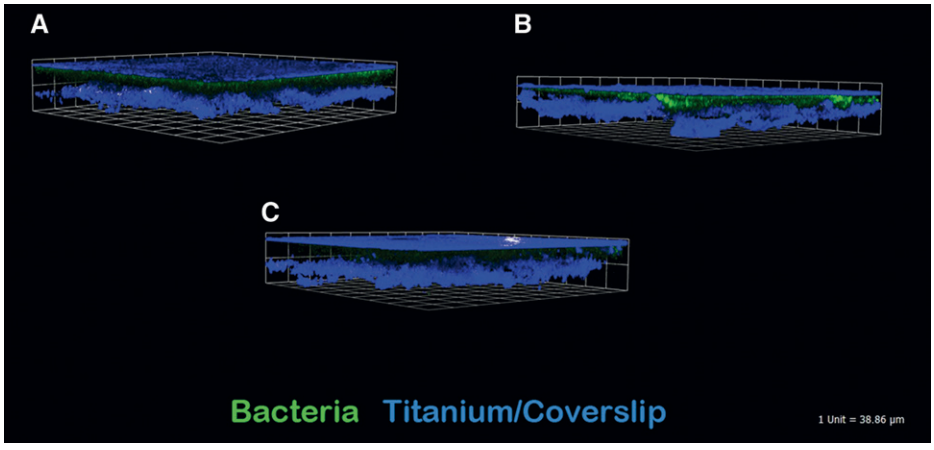

Figure 2.
Three-dimensional reconstruction of S. aureus *biofilm by CLSM. Biofilms were grown for 72 hours and then treated with glycine and erythritol-chlorhexidine. Biofilms were stained with a fluorescent stain consisting of a mixture of two dyes: green represents the viable cells; propidium iodide (red) represents the dead cells.* ***A)*** *Biofilm after mechanical treatment control.* ***B)*** *Biofilm after glycine air polishing.* ***C)*** *Biofilm after erythritol-chlorhexidine air polishing; note the reduction of fluorescence intensity.*

demonstrated by MIC test. In particular, the combination of erythritol-chlorhexidine seemed to display stronger activity than glycine against all microbial strains tested. In addition, MMC tests suggested that, at the concentrations tested, glycine has a bacteriostatic effect, whereas erythritol-chlorhexidine also display bactericidal activity. Glycine seemed to be less effective against *S. aureus* than against *B. fragilis* and *C. albicans*. After glycine treatment, the reduction of surviving cells into the residual biofilm was ≈30% of both *B. fragilis* and *C. albicans* compared with 15% for *S. aureus*. The results of the semiquantitative analysis by spectrophotometric assay indicated that the treatment with erythritol-chlorhexidine was effective in detaching biofilm from titanium, as a single treatment induced a mean biofilm reduction of almost 65% for all microbial strains tested. In contrast, glycine was effective against *B. fragilis* and *C. albicans*, but not against *S. aureus*, confirming the results observed in microbial recovery. CLSM images of *S. aureus* biofilm display this difference between the two treatments (Fig. 2). In future studies it would be of interest to also perform a quantitative CLSM analysis. To obtain reliable data, mechanical controls (disks treated with a water jet only) are used in this study. However, previous studies have found no reduction in viable bacterial counts after subgingival irrigation with water alone, suggesting that the water jet emerging from the tip of the handpiece in itself may not be sufficient in removing subgingival biofilm.[50] In addition, in the present study, the treatment is performed at a distance of 20 mm, aiming at minimizing the mechanical effect of the spray flow on biofilm structure. The instrumentation time is limited to 5 seconds. These expedients are used to limit the bias due to mechanical stress caused by air polishing. Mechanical controls are air polished with a flux of water alone, under the same experimental conditions.

CONCLUSIONS

In conclusion, air polishing with erythritol-chlorhexidine seems to be a valuable alternative to the traditional glycine treatment. The combination of an antimicrobial substance, such as chlorhexidine, with an antibiofilm substance, such as erythritol, may help to overcome the limitations of the chemical control of biofilm-associated infections. To improve these

observations, subsequent studies might be necessary, in which a larger sample size and a wider spectrum of oral microorganisms and substrate surfaces (e.g., dentin and/or enamel) are tested. In addition, it would be interesting to study the potential to prevent biofilm formation (i.e., by treating substrates before allowing biofilm growth). Finally, the in vivo efficacy of the new treatment should be investigated in clinical studies.

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