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ORIGINAL ARTICLE

Photodynamic inactivation of *Streptococcus mutans* and *Streptococcus sanguinis* biofilms in vitro

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Abstract The purpose of this study was to evaluate specific effects of photodynamic inactivation (PDI) using erythrosine (ER) and Rose Bengal (RB) photosensitizers and a blue light-emitting diode (LED) on the viability of *Streptococcus mutans* and *Streptococcus sanguinis* biofilms. Biofilms were grown in acrylic disks immersed in broth to production of biofilms, inoculated with microbial suspension (10^6 cells/mL) and incubated for 48 h. After the formation of biofilms, the effects of the photosensitizers ER and RB at a concentration of 5 μM for 5 min and blue LED (455±20 nm) for 180 s, photosensitizers alone and conjugated were evaluated. Next, the disks were placed in tubes with sterile physiological solution (0.9 % sodium chloride) and sonicated for to disperse the biofilms. Tenfold serial dilutions were carried and aliquots seeded in brain heart infusion agar which were then incubated for 48 h. Then the numbers colony-forming units per milliliter (CFU/mL; $\log_{10}$) were counted and analyzed statistically (ANOVA, Tukey test, $P \leq 0.05$). Significant decreases in the viability of all microorganisms were observed for biofilms exposed to PDI mediated by both photosensitizers. The reductions with RB and ER were, 0.62 and 0.52 $\log_{10}$ CFU mL^{-1} for *S. mutans* biofilms ($p=0.001$), and 0.95 and 0.88 $\log_{10}$ CFU mL^{-1} for *S. sanguinis* biofilms ($p=0.001$), respectively. The results showed that biofilms formed in vitro by *S. mutans* and *S. sanguinis*, were sensitive to PDI using a blue LED associated with photosensitizers ER or RB, indicating its use in the control of caries and periodontal diseases.

Keywords Biofilm · *Streptococcus mutans* · *Streptococccus sanguinis* · Photodynamic inactivation

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Introduction

Biofilm cells are organized into structured communities enclosed within a matrix of extracellular material. The human oral cavity is inhabited by more than 500 species of bacteria, which are organized in biofilms [1].

Approximately 20 % of the oral bacteria are streptococci [2]. The oral streptococci pioneer early dental plaque formation and have a specific temporal and spatial distribution that is crucial for the development of oral biofilms [3]. *Streptococcus mutans* is considered a major pathogen causing human dental caries (also known as tooth decay) [4]. *S. mutans* normally exists as a regular member of the mature dental biofilm community; however, under certain conditions, it can become dominant to cause dental caries [5]. *Streptococcus sanguinis* is also a member of the oral biofilm community [6], recognized not only for its historical association with life-threatening bacterial endocarditis, but also because of its putative antagonistic role in dental caries [7]

and periodontal diseases [8]. In terms of the former, *S. sanguinis* may compete with the *S. mutans* for colonization sites on tooth surfaces, since both groups of bacteria require the presence of teeth for colonization [9] and may exhibit direct biochemical antagonism in situ [10].

Many strategies for biofilm control have been proposed, including stopping biofilm growth, blocking biofilm attachment, killing biofilms, promoting detachment, and mechanical removal [11, 12]. Among these approaches, application of chemical additives or biocides to inhibit microbial growth or metabolism of biofilms is the most common and economical method. However, biofilms are infamous for their recalcitrance to antimicrobial agents. Many scientists have attempted to develop new antimicrobial treatment approaches that do not induce bacterial resistance to antimicrobial agents. Using analogs of quorum-sensing signal molecules to block cell-to cell communication within biofilms was deemed promising, although much research is still required [13]. A possible alternative to reduce biofilms is the photodynamic inactivation (PDI).

In PDI, the interaction between light and certain photoactive compounds, known as photosensitizers, is used to inactivate cell functions [14]. When a photosensitizer is irradiated with light of an appropriate wavelength and at a certain level, the molecule becomes excited and consequently experiences a series of molecular energy transfers. These energy transfers lead to the production of cytotoxic products, including singlet oxygen and free radicals [15, 16]. These products are capable of damaging essential components of the cells or modifying metabolic activities in an irreversible way, which may result in cell death [17, 18].

The photosensitizers of the xanthene group, rose bengal (RB), and erythrosine (ER), are cyclic compounds that contain three aromatic rings in a linear arrangement and an oxygen atom in the center of the ring, which absorbs light in the visible spectrum. RB is a halogenated derivative of fluorescein, which is used in ophthalmology for the diagnosis of ophthalmological diseases. Its photodynamic mechanism consists of the formation of 80 % singlet oxygen and 20 % superoxide anion [19]. The photodynamic action of RB on Gram-positive and Gram-negative bacteria has been demonstrated in some studies [19–24]. ER is a cyclic compound that absorbs light in the visible region (500–550 nm) and is therefore able to initiate photochemical reactions. Its main application in dentistry is the demonstration of the presence of dental biofilm [25, 26].

Although PDI of planktonic cultures has been studied for many years, little is known about using PDI against biofilm cultures. In this study, we examined the effect of PDI using exogenous photosensitizers, ER and RB, and light-emitting diode (LED) on *S. mutans* and *S. sanguinis* biofilms.

Materials and methods

Microorganisms and production of biofilms

The biofilms formation was developed as methodology proposed by Pereira et al. [27]. Two reference strains [American Type Culture Collection (ATCC)] of *Streptococcus* ssp. maintained in our laboratory stock collection were included in the study. Standard suspensions of *S. mutans* (ATCC 35688) and *S. sanguinis* (ATTC 10556) containing 10^6 viable cells/ml each were prepared. For this purpose, *S. mutans* and *S. sanguinis* was seeded onto brain heart infusion (BHI) agar (Difco, Detroit, MI, USA) and incubated for 48 h. All incubations were carried out at 37 °C and at a partial pressure of 5 % CO_2. After incubation, the growth was suspended in sterile physiological solution [0.9 % sodium chloride (NaCl)] and the number of viable cells in suspension was counted in a spectrophotometer (B582, Micronal, São Paulo, Brazil). The parameters of optical density and wavelength used were, respectively, 0.620 and 398 nm. These parameters were previously established by means of a standard curve with colony-forming units (CFU) vs. absorbance. The broth used for production of biofilms was proposed by Gibbons; Nygaard [28] and contains 20 g tripticase, 2 g NaCl, 3 g K2HPO4, 2 g KH2PO4, 1 g K2CO3, 120 mg MgSO4, 15 mg MnSO4, and 50 g C6H8O7 dissolved in 1000 mL of distilled water. The broth was sterilized by autoclaving at 121 °C for 15 min.

The biofilms were grown in 120 sterile acrylic resin disks (06 mm diameter; Clássico, São Paulo, Brazil), 60 each microorganism tested: *S. mutans* and *S. sanguinis*. The disks were placed in plates of 24 wells (Costar Corning, New York, EUA) with 2 mL sterile broth. The wells were inoculated only with 0.1 mL of *S. mutans* or *S. sanguinis* standard suspensions. The plates were incubated for 48 h.

Photosensitizer and light source

RB and ER in powder (Aldrich Chemical Co., Milwaukee, WI, USA) at a concentration of 5 μM each, were used for the sensitization of biofilms. Each photosensitizer solution was prepared by dissolution of the dye in physiological solution (0.9 % NaCl) and filtration through a sterile 0.22 μm Millipore membrane (São Paulo, Brazil). After filtration, the photosensitizer solutions were stored in the dark.

A blue LED (MMOptics, São Carlos, Brazil) with a wavelength range of 455±20 nm, an output power of 200 mW, a fluence of 95 Jcm^{-2} (energy of 36 J and time at 180 s) and a fluence rate of 526 mW cm^{-2} was used as a light source [29]. The temperature at the plate—24 wells (Costar Corning, New York, USA) was monitored using an infrared thermometer (MX4; Raytek, Sorocaba, Brazil); no increases in temperature.

For the each photosensitizer, the biofilms were submitted to four experimental conditions: ER+L+ or RB+L+ LED irradiation using ER or RB as photosensitizer (n=10); RB+L− or ER+L−, treatment with ER or RB alone (n=10); P−L+, LED irradiation alone (n=10); and P−L−, not submitted to LED irradiation or photosensitizer treatment.

Photodynamic inactivation of biofilms

After 48 h of incubation, the disks containing the biofilms were aseptically transferred to the second and third rows of the plate—24 wells—and washed twice with sterile physiological solution (0.9 % NaCl), in order to remove loosely bound material. Following this, AC disks containing the biofilms were placed in the fourth row of the plate—24 wells

According to the experimental conditions described 0.1 mL of the photosensitizer was added for groups RB+L+ and RB+L− or ER+L+ and ER+L−, whereas 0.1 mL physiological solution (0.9 % NaCl) was added for groups P−L+ and P−L−, and subsequently left in the dark for 5 min (pre-irradiation time). The biofilms of groups P−L+, RB+L+ and ER+L+ was then irradiated according to the protocol described. Irradiation was performed under aseptic conditions under a laminar flow hood in the dark.

After PDI, the disks were placed in tubes with 10 mL of sterile physiological solution (0.9 % NaCl) and sonicated (Sonoplus HD 2200, 50 W, Bandelin Eletronic) for 30 s to disperse the biofilms. Tenfold serial dilutions were carried and aliquots of 0.1 mL were seeded in duplicate onto BHI agar and incubated for 48 h. After 48 h of incubation, the number of CFU per milliliter (CFU/mL) was determined. The results were log-transformed ($\log_{10}$) and analyzed by analysis of variance and the Tukey test. A P value of ≤0.05 was considered to indicate a statistically significant difference.

Results

PDI mediated by 5 μM photosensitizer erythrosine of biofilms resulted in 0.52 and 0.88 $\log_{10}$ CFU mL^{-1} reductions of *S. mutans* and *S. sanguinis*, respectively (Fig. 1). The differences for the ER+L+ groups of both species were statistically significant relative to the remaining groups (P−L−) with P values relative to the control group of 0.001 for *S. mutans* and *S. sanguinis* biofilms.

The assays of PDI with photosensitizer Rose Bengal also promoted reductions statistically significant relative to the remaining groups (P−L−), with P values relative to the control group of 0.001 for *S. mutans* and *S. sanguinis*

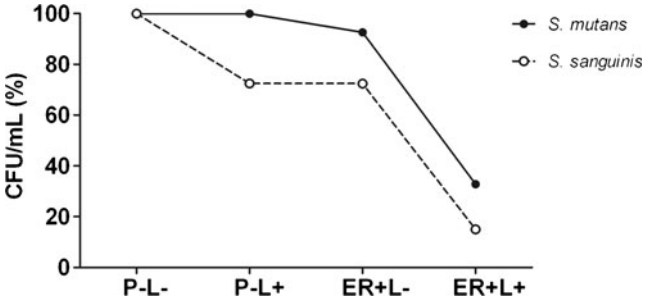

Fig. 1 Percentage of reduction, expressed as mean values (colony-forming units per milliliter), in the viability of *S. mutans* and *S. sanguinis* biofilms exposed to erythrosine photosensitizer (ER+L−), LED (P−L+) or both erythrosine photosensitizer and LED (ER+L+) relative to the control group (P−L−)

biofilms. The reductions were, respectively, 0.62 and 0.95 $\log_{10}$ CFU mL^{-} for *S. mutans* and *S. sanguinis* biofilms (Fig. 2).

Discussion

A series of advantages found for microbial colony can be attributed to the biofilms. There is a better communication between cells, due to their continuity, which certainly favors biochemical activities and greater proliferation, easier access to niches and resources. These could not be used by individual cells due to the group defense against microorganisms by saliva and antimicrobial agents [30]. The limited access of topical agents to oral plaque biofilms and the development of antibiotic resistance have led to the necessity for alternative strategies to control biofilms and to treat periodontal diseases [31]. Therefore, it has been essential to investigate the action of PDI in the control of biofilms.

PDI is mediated by singlet oxygen which has a direct effect on extracellular molecules. Thus, the polysaccharides present in the extracellular matrix of the polymers of a bacterial biofilm are also susceptible to photodamage [31].

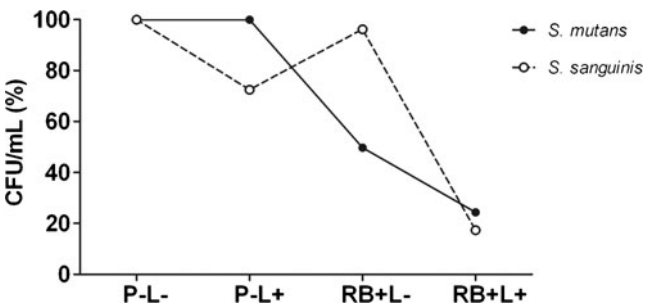

Fig. 2 Percentage of reduction, expressed as mean values (colony-forming units per milliliter), in the viability of *S. mutans* and *S. sanguinis* biofilms exposed to rose bengal photosensitizer (RB+L−), LED (P−L+) or both rose bengal photosensitizer and LED (RB+L+) relative to the control group (P−L−)

Such dual activity is not exhibited by antibiotics and may represent a significant advantage of PDI. Moreover, development of resistance to the cytotoxic action of singlet oxygen or free radicals seems unlikely. PDI is effective against both antibiotic-resistant and susceptible bacteria. Moreover, repeated photosensitization procedures have not induced the selection of resistant strains [32] and bacterial resistance could not be generated in an experimental study protocol [33].

In the present study, we investigated the antimicrobial photodynamic effect of a LED associated with ER or RB on *S. mutans* and *S. sanguinis* biofilms, in vitro. The choice of a LED, instead of a lasers device, was due to their wider emission bands, smaller size, reduced weight and cost, greater flexibility in treatment irradiation time and easy operation [31, 34]. LEDs are used in dentistry as bleaching tools that do not damage oral tissues. LEDs have shown potent activity in PDI and lack of absence of antimicrobial action alone [35, 36].

The results presented here demonstrate that of ER and RB photosensitizer, irradiated by blue LED, exerted a significant photodynamic effect on *S. mutans* and *S. sanguinis* biofilms. The reductions with RB and ER were, respectively, 0.62 and 0.52 $\log_{10}$ CFU mL^{-1} for *S. mutans* biofilms (p=0.001) and 0.95 and 0.88 $\log_{10}$ CFU mL^{-1} for *S. sangui*nis biofilms (p= 0.001). Others studies evaluated the photodynamic effect of ER associated with a light source in *S. mutans* biofilms [26]. Moreover, we did not find in the literature any papers describing the effect of PDI with ER or RB and a blue LED on *S. mutans* and *S. sanguinis* biofilms.

Wood et al. [26] evaluated the photodynamic activity of ER, methylene blue and photophrin irradiated with a tungsten-filament lamp on *S. mutans* biofilms. ER (22 μM) was found to be the most effective photosensitizer, resulting in a 2.2 $\log_{10}$ reduction for 24 h biofilms and a 3.0 $\log_{10}$ reduction for 288 h biofilms. Metcalf et al. [25] demonstrated that fractionation of white light during irradiation of *S. mutans* biofilms treated with 22 μM ER increased bacterial killing by 1.7 $\log_{10}$ when compared to continuous light irradiation. The most photodynamic efficacy achieved in these studies, may be due to a higher concentration of the erythrosine photosensitizer and different sources light used.

PDI, with ER and RB photosensitizers, has been more effective in planktonic culture of *S. mutans*. In the study by Rolim et al. [24], with planktonic cultures, the ER photosensitizer (163.5 μM) had a significant effect on the viability of *S. mutans*, while RB photosensitizer (163.5 μM) eliminated 100 % *S. mutans*, when irradiated with a blue handheld photopolymerizer. Costa et al. [23] demonstrated that PDI performed using a blue LED was able to obtain a reduction of 5.16 and 6.86 $\log_{10}$ CFU mL^{-1} with ER and RB photosensitizers (2 μM), respectively, on planktonic cultures of *S. mutans*. Paulino et al. [19] also demonstrated the photodynamic activity of RB on planktonic cultures of *S. mutans*, with a concentration of 0.5 μM of the photosensitizer resulting in a 3 $\log_{10}$ reduction of cells irradiated with a handheld photopolymerizer. Other study that evaluated the efficacy of the PDI was realized by Bolean et al. [22], when 0.1 μM of RB photosensitizer, irradiated with light, promoted 100 % of reduction planktonic of cultures of *S. mutans*.

The *S. sanguinis* biofilms were more sensible to PDI, with both photosensitizers, that *S. mutans* biofilms. PDI in *S. sanguinis* biofilms has not been previously studied. Therefore, the results of this investigation have been of extreme relevance due to the significant reductions achieved. In the literature, PDI was evaluated in planktonic cultures of *S. sanguinis* only [37], with the photosensitizer toluidine blue-O irradiated by AlGaInP diode laser. *S. sanguinis* is a member of the viridans group of streptococci and a primary colonizer of the human oral cavity, due to the recognition of specific receptors of the acquired pellicle on dental enamel surface [38, 39]. The carious lesions produced by *S. sanguinis* occur mostly in grooves and are significantly smaller than those produced by *S. mutans* [40]. Furthermore, it is implicated in endocarditis, an infection of the valves or lining of the heart, after dental work or in severe periodontal disease [41].

PDI performed with RB in the absence of Led irradiation had a significant effect on the viability of *S. mutans* biofilms (0.34 $\log_{10}$ CFU mL^{-1}), when compared to control group, where neither light nor photosensitizer was applied. A recent study, conducted by Rolim et al. [24], demonstrated that PDI performed using RB, alone and in the presence of light, was able to promote complete reduction in microbial viability of *S. mutans* planktonic cultures. One possible explanation for this result is that these authors used a high concentration of RB photosensitizer (163.5 μM), resulting in bacterial toxicity. Paulino et al. [19] investigated the toxicity of RB at concentrations of 0–10 μM to *S. mutans* at a cell density of 10^3 CFU/mL, and they showed that RB is not toxic at concentrations lower than 5.0 μM. Furthermore, the latter study, the same authors observed that the application of RB concentrations lower than 5.0 μM in the dark were also not toxic to fibroblasts [42]. In another study, performed by Costa et al. [23], confirms these results, where a low concentration of RB photosensitizer (2 μM) did not present cytotoxicity, in the absence of light, on planktonic cultures of *S. mutans*.

The biofilms of *S. mutans* and *S. sanguinis* treated with ER photosensitizer, in the absence of Led irradiation, did not show significant reductions. ER is used in dental practice for the detection of dental biofilms at concentrations of 9–25 mM [43], higher than the concentrations used in this study. ER presents advantages over other photosensitizers since it is not toxic to the host and has already been approved for use in dentistry [26, 44].

In the relation to isolated effects of the LED irradiation (L+ P− group), significant reductions in the numbers of CFU/mL

from the biofilms were not observed. These data results are in agreement with the ones reported in previous investigations, in where LEDs have shown potent activity in PDI and lack of absence of antimicrobial action alone [35, 36].

Additionally, the divergent results found in our study might have occurred due to lack of a predefined protocol for PDI use. The great variety of biofilm models, concentration, period of incubation, type of photosensitizer, as well as, the physiological state of microorganisms, exposure time, and the light energy density, might also influence PDI results [45].

Based on the current results, it could conclude that biofilms formed in vitro by *S. mutans* and *S. sanguinis*, were sensitive to PDI using a blue LED associated with photosensitizers ER or RB, indicating its use in the control of caries and periodontal diseases.

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