Effect of photodynamic therapy on clinical isolates of *Staphylococcus* spp

Michelle Miyabe[a]
Juliana Campos Junqueira[a]
Anna Carolina Borges Pereira da Costa[a]
Antonio Olavo Cardoso Jorge[a]
Martha Simões Ribeiro[b]
Ilíria Salomão Feist[b]

[a] Department of Biosciences and Oral Diagnosis, School of Dentistry of São José dos Campos, UNESP - Univ. Estadual Paulista, São José dos Campos, SP, Brazil.

[b] Center for Lasers and Applications, Institute of Energetic and Nuclear Researches - IPEN-CNEN/SP, Universidade de São Paulo - USP, São Paulo, SP, Brazil.

Abstract: *Staphylococcus* spp. are opportunistic microorganisms known for their capacity to develop resistance against antimicrobial agents. The objective of this study was to evaluate the effect of photodynamic therapy (PDT) on 20 *Staphylococcus* strains isolated from the human oral cavity, including *S. aureus*, *S. schleiferi*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, and *S. lentus*. A suspension of each *Staphylococcus* strain (10^6 cells/mL) was submitted to PDT using methylene blue and a low power laser. The isolated effects of methylene blue, laser treatment and ciprofloxacin were also evaluated. After the experimental treatments, 0.1 mL aliquots of the suspensions were seeded onto BHI agar for determination of the number of colony-forming units (CFU/mL). The results were analyzed by analysis of variance and Tukey's test ($p \leq 0.05$). The mean reduction in bacterial counts of the strains submitted to PDT ranged from 4.89 to 6.83 CFU ($\log_{10}$)/mL, with the observation of a decreasing susceptibility to treatment of *S. schleiferi*, *S. haemolyticus*, *S. epidermidis*, *S. capitis*, *S. aureus*, and *S. lentus*. The results showed that PDT was effective in reducing the number of viable cells of all clinical *Staphylococcus* isolates studied.

Descriptors: *Staphylococcus*; Photochemotherapy; Methylene Blue; Ciprofloxacin.

Introduction

Several species of the genus *Staphylococcus* can be isolated from the human oral cavity and are part of the transient microbiota.[1] These microorganisms are found in the oral cavity of approximately 95% of healthy individuals and have become a matter of concern because of their capacity to develop resistance against antimicrobial agents.[2,3] In addition to their involvement in oral diseases, such as angular cheilitis, parotiditis, staphylococcal mucositis and periodontitis, these bacteria are the causative agents of severe skin and soft tissue infections that are associated with an increased risk of complications, such as bacteremia, pneumonia and endocarditis.[1,4-7]

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from the oral cavity of healthy individuals, a finding that indicates an increased risk of dissemination to other sites.[7,8] Other *Staphylococcus* species have also been recognized as important etiological agents of opportunistic infections in individuals with differing predisposing factors, and some isolates have shown a reduced susceptibility to β-lactam antibiotics.[3,9] Vancomycin is the antibiotic of choice in the case of reduced

Corresponding author:
Anna Carolina Borges Pereira da Costa
Universidade Estadual Paulista (UNESP)
Av. Engenheiro Francisco José Longo, 777, São Dimas
São José dos Campos - SP - Brazil
CEP: 12245-000
E-mail: *carol_biolog@yahoo.com.br*

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β-lactam susceptibility. However, although resistance is rare, therapeutic failure is increasingly being reported.[4]

Photodynamic therapy (PDT) has emerged as a therapeutic option for the treatment of infectious diseases. This therapy consists of the activation of a photosensitive dye with light of an appropriate wavelength, consequently resulting in the production of reactive oxygen species, such as free radicals and singlet oxygen. These reactive oxygen species can damage DNA and the cell membrane, resulting in the leakage of cell components, inactivation of transport systems and cell death.[10,11]

The objective of the present study was to evaluate the effect of photodynamic antimicrobial chemotherapy on different *Staphylococcus* strains isolated from the human oral cavity.

Materials and Methods

Staphylococcus strains

The study was approved by the Ethics Committee of the School of Dentistry of São José dos Campos, UNESP (07/2007-PH/CEP).

Twenty *Staphylococcus* strains isolated from the human oral cavity (*S. aureus*, n = 5; *S. schleiferi*, n = 5; *S. epidermidis*, n = 6; *S. capitis*, n = 2; *S. haemolyticus*, n = 1, and *S. lentus*, n = 1) were studied. These strains were obtained from the Laboratory of Microbiology and Immunology, School of Dentistry of São José dos Campos, UNESP.

Twenty-four-hour cultures, incubated at 37°C on mannitol agar (Difco, Detroit, MI, USA), were inoculated into brain-heart infusion broth (Difco) and incubated for a further 18 h at 37°C. The cultures were then centrifuged at 1300 x *g* for 10 min, the supernatant was discarded, and the cell pellet was resuspended in 5 mL sterile saline (0.85% NaCl). This procedure was repeated once. The suspension was standardized in a spectrophotometer (B582, Micronal, São Paulo, Brazil) to an optical density of 0.374 at a wavelength of 490 nm, corresponding to 10^6 cells /mL.

Photosensitizer, laser and antibiotic treatment

Methylene blue (MB), at a concentration of 3 mM, was used for photosensitization of the *Staphylococcus* strains. The dye solution was prepared by dissolving the powder (Sigma-Aldrich, Steinheim, Germany) in 0.85% NaCl and filtering it through a 0.22-μm membrane filter (Millipore, São Paulo, Brazil). After filtration, the dye solution was stored in the dark.

A gallium-aluminum-arsenide laser (Easy Laser, Clean Line, Taubaté, Brazil), emitting light at 660 nm (visible red), was used as the light source. The wavelength of the laser corresponds to the maximum absorption of MB. The laser settings were as follows: power of 35 mW, energy of 10 J, time of 285 s, fluence rate of 92 mW/cm², and fluence of 26.3 J/cm².

Ciprofloxacin (Vivência Compounding Pharmacy, Pindamonhangaba, SP, Brazil) was used as an antibiotic at a concentration of 1.6 mg/mL.

In vitro photosensitization

Thirty assays were carried out for each suspension for a total of 600 assays. The *Staphylococcus* spp. strains were submitted to the following experimental conditions (n = 6):

a. photosensitization with MB and laser irradiation (P+L+);
b. photosensitization with MB in the absence of light (P+L–);
c. treatment with saline and laser irradiation (P–L+);
d. treatment with the antibiotic (AB); and
e. treatment with saline in the absence of light as a control group (P–L–).

Sterile 96-well flat-bottom microtiter plates with a lid (Costar Corning, New York, USA) were used. An aliquot (0.1 mL) of the bacterial suspension was added to each well (area of 0.38 cm²). Next, 0.1 mL of the photosensitizer was added for groups P+L+ and P+L–, 0.1 mL of saline for groups P–L+ and P–L–, and 0.1 mL of the antibiotic for group AB. The plates were then shaken for 5 min in an orbital shaker (Solab, Piracicaba, Brazil). After this period, the contents of the wells of groups P+L+ and P–L+ was irradiated as described above. The samples were irradiated under aseptic conditions in a laminar flow chamber in the dark.

After the experimental treatments, serial dilutions were prepared and 0.1-mL aliquots of the dilutions were seeded in duplicate onto brain-heart infusion agar plates (Difco). After incubation for 24 h at 37°C, the number of colony-forming units (CFU/mL) was determined, and the results were log-transformed (log_{10}).

Statistical analysis

The results (log_{10} CFU/mL) were analyzed by analysis of variance (ANOVA) and the Tukey test, with the level of significance set at 5%, using the Minitab 14.0 program.

Results

Figures 1 and 2 show the mean and standard deviation of CFU/mL obtained for the different *Staphylococcus* species studied under each experimental condition. A significant reduction in bacterial counts was observed for the groups submitted to PDT (P+L+) when compared to the other groups (P–L–, P–L+ and P+L–). Ciprofloxacin treatment resulted in a reduction of 100% for all the groups except for *S. schleiferi*, which presented a reduction of 5.97 log_{10} CFU/mL.

A small reduction in bacterial counts was observed for the P+L– groups in comparison to the P–L– groups for all species. However, the difference was only significant for two species: *S. epidermidis* and *S. haemolyticus*. The number of bacterial cells was higher in the P–L+ groups compared to the P–L– groups, but the difference was not significant.

Table 1 shows the log_{10} CFU/mL reduction observed for the P+L+ groups compared to the P–L– groups. Among the strains tested, *S. lentus* was the species most resistant to PDT, followed by *S. aure-*

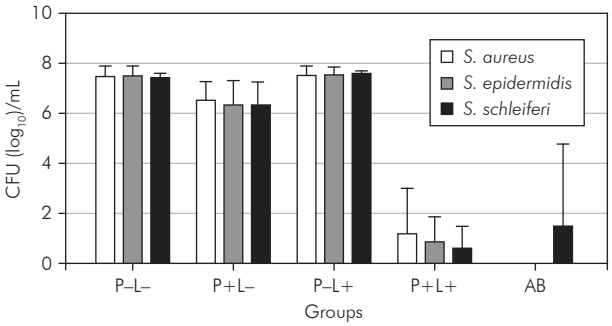

Figure 1 - Mean and standard deviation of log_{10} CFU/mL obtained for *S. aureus*, *S. epidermidis* and *S. schleiferi* under the different experimental conditions. Experimental groups: photosensitizer and laser (P+L+), photosensitizer alone (P+L–), laser alone (P–L+), saline (P–L–), and antibiotic treatment (AB).

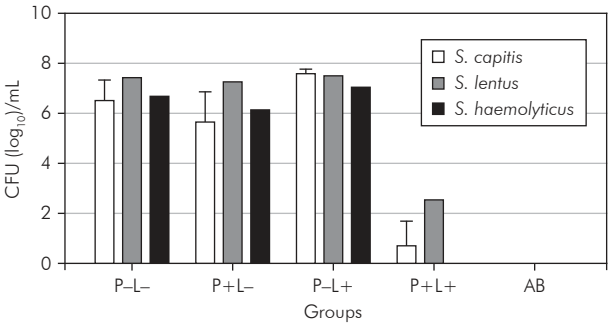

Figure 2 - Mean and standard deviation of log_{10} CFU/mL obtained for *S. capitis*, *S. lentus* and *S. haemolyticus* under the different experimental conditions. Experimental groups: photosensitizer and laser (P+L+), photosensitizer alone (P+L–), laser alone (P–L+), saline (P–L–), and antibiotic treatment (AB).

Table 1 - Mean log_{10} CFU/mL of *Staphylococcus* spp. under the different experimental conditions and log_{10} reduction in the number of cells for the groups submitted to photodynamic therapy compared to the control groups. P+L+: photosensitizer and laser; P+L–: photosensitizer alone; P–L+: laser alone; P–L–: saline (control group).

Strains	CFU (log_{10})/mL				
	P–L–	P–L+	P+L–	P+L+	Reduction in log of P+L+
S. aureus (n = 5)	7.46^{A}	$7,51^{A}$	6.51^{A}	1.19^{B}	6.27
S.epidermidis (n = 6)	7.49^{A}	7.53^{A}	6.33^{B}	0.86^{C}	6.63
S. schleiferi (n = 5)	7.44^{A}	7.59^{A}	6.32^{A}	0.61^{C}	6.83
S. capitis (n = 2)	7.10^{A}	7.60^{A}	5.68^{A}	0.71^{B}	6.39
S. haemolyticus (n = 1)	6.71^{A}	7.06^{A}	6.15^{B}	0.00^{C}	6.71
S. lentus (n = 1)	7.44^{A}	7.53^{A}	7.26^{A}	2.55^{B}	4.89
Mean					6.29

A, B and C: statistically significant difference (Tukey's test, $p \leq 0.05$).

us, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, and *S. schleiferi*.

Discussion

In the present study, PDT resulted in a mean reduction of 6.29 $\log_{10}$ CFU/mL in comparison to the control group (P–L–), a reduction greater than reported in some other studies.[12-14] Grinholc *et al.*[12], treating MRSA and methicillin-sensitive *S. aureus* (MSSA) strains with 25 μM protoporphyrin irradiated with 624 nm light, observed a reduction of 0 to 3 log for MRSA and of 0.2 to 3 log for MSSA, demonstrating a similar sensitivity to PDT for β-lactam-resistant and -sensitive strains. Wainwright *et al.*[13] studied the effect of phenothiazine dyes irradiated with visible light (350-800 nm) on an *S. aureus* reference strain and on MRSA. The reduction in bacterial counts achieved with PDT was 4 $\log_{10}$ when 5 μM MB was used for the reference strain and 10 to 50 μM MB for MRSA. In another study, Tubby *et al.*[14] were able to kill approximately 6 log MRSA cells with PDT, using 20 μM MB and a laser light dose of 9.65 J/cm². In addition, PDT reduced the activity of V8 protease and α-hemolysin by 100% and the activity of sphingomyelinase by 92%. The authors suggested that the reactive oxygen species formed during PDT interact with, and inactivate, bacterial virulence factors, providing an additional mechanism for the antibacterial action of this therapy.

The formation of a biofilm, observed in *Staphylococcus* strains, increases their resistance to antibiotics and protects the cells against the action of the immune system.[15] Sharma *et al.*[16] studied the effects of PDT on biofilm formation in MRSA and *S. epidermidis* and observed that toluidine blue, combined with a diode laser, showed low antibacterial activity. In addition, *S. aureus* was more resistant to PDT than *S. epidermidis*.

Zolfaghari *et al.*[17] evaluated the *in vivo* effect of PDT in mice with excisional and superficial skin wounds inoculated with a suspension of MRSA. The wounds were treated with 0.3 μM MB and irradiated with 360 J/cm² of diode laser light for 30 min. Treatment resulted in a reduction of 1.4 $\log_{10}$ CFU/mL in excision wounds and of 1.15 $\log_{10}$ CFU/mL in superficial wounds. The authors emphasized the importance of these results because they represent the first report of the *in vivo* killing of MRSA and raise the possibility of attempting to reproduce this treatment in humans.

In the present study, administration of the dye alone exerted a low antimicrobial effect, with a significant difference being observed only for *S. epidermidis* and *S. haemolyticus*. A dye concentration of 3 mM was used, which exhibits low toxicity.[18-20] Gois *et al.*[21] also reported a reduction in the number of *S. aureus* cells of 0.97, 0.97 and 1.05 $\log_{10}$ when irradiated with red LED (20 J/cm²) and diode laser light (20 and 40 J/cm²), respectively, in the absence of a photosensitizer.

Ciprofloxacin used in the present study is a broad spectrum antibiotic with potent activity against bacteria of the genus *Staphylococcus*.[22] This antibiotic killed all of the strains studied, except for one *S. schleiferi* isolate. This latter finding may be due to previous exposure of this microorganism to the antibiotic or to the development of primary resistance. However, among the *Staphylococcus* species submitted to PDT, *S. schleiferi* was the most sensitive, with a mean reduction of 6.83 $\log_{10}$ CFU/mL, suggesting that PDT might be used as an alternative for the treatment of antibiotic-resistant strains.

Conclusion

PDT was effective in reducing the number of viable cells of all clinical *Staphylococcus* isolates studied.

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