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Sonophotodynamic Therapy for the inactivation of *Staphylococcus aureus* biofilm

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ABSTRACT

Antimicrobial Photodynamic Therapy (aPDT) has been investigated as an alternative method for the inactivation of microorganisms. This treatment, which is based on the application of a photosensitizer and visible light, has a reduced effectiveness when the microorganisms are organized as biofilm. Recently, Sonodynamic Therapy (SDT) has also been suggested as an antimicrobial treatment presenting the advantage of activating photosensitizer by the use of ultrasound (US), which propagates deeper into the tissue and is able to disrupt the biofilm. In this sense, this study aimed to evaluate the efficacy of associating US with aPDT mediated by curcumin (Cur), in order to disrupt *Staphylococcus aureus* biofilms and increase the inactivation of the bacteria. For this, standardized suspensions of *S. aureus* were prepared (10^8) and after 48 h of biofilm formation, samples received the following treatments: aPDT (Cur and blue LED light), SDT (Cur and US) and SPDT (incubation with Cur and, then, simultaneously application of US and light). Additional samples received Cur, light or US only, or no treatment (control). To determine cell survival, the biofilms were removed and aliquots were serially diluted and plated in Brain Heart Infusion Agar. After 24 h of incubation at 37°C, the colony forming units were calculated. The preliminary results demonstrated that US in combination with aPDT (SPDT) showed higher and significant bacteria reduction compared to the application of SDT and aPDT. Cur, LED light or US alone did not have any effect. This result highlights the enhanced effect of ultrasound and aPDT against *S. aureus* biofilms.

Keywords: Antimicrobial photodynamic therapy, ultrasound, sonodynamic therapy, curcumin, *Staphylococcus aureus*

1. INTRODUCTION

Staphylococcus aureus, a gram-positive bacteria, is often found on the skin and oropharyngeal tract of healthy individuals and exhibits a remarkable ability to adapt quickly to environmental changes[1]. Even under adverse conditions, this bacteria is able to cause a variety of infections ranging from superficial skin infections to pneumonia and sepsis[2]. Studies have shown that this bacteria is frequently isolated from saliva and dental prosthesis and, when *S. aureus* is aspirated by the individual, this is a common route for pneumonia infection[3]. In the United States, this bacteria was considered to be the second cause of hospital bacteraemia[4]. The capacity of *S. aureus* to form biofilm is an important virulence factor, which is involved with the persistence and pathogenicity of the bacteria[5]. Biofilms are organized, structured microbial communities surrounded by an extracellular matrix (ECM) produced by the microorganisms, which promotes greater resistance of the bacteria to the antibiotics, as it limits the penetration of the drugs and protects the cells against the immune response of the host[5]. Besides that, the development of resistance to antibiotics may also be involved to the acquisition of determinants by horizontal gene transfer of mobile genetic elements or by mutations that alter the drug binding sites on molecular targets and by increasing expression of endogenous efflux pumps[6].

Antimicrobial Photodynamic Therapy (aPDT) emerged as an alternative method of microbial inactivation, which is based on the application of a photosensitizer (PS) followed by the irradiation of a light in an appropriated wavelength. The light activates the PS and, in the presence of the oxygen, there is the production of reactive oxygen species (ROS)[7]. Studies have shown that this method is effective to eradicate *S. aureus* suspension, using a variety of PS[8,9,10,11,12].

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However, when the bacteria is organized in biofilm, complete killing has not been observed yet. Sonodynamic Therapy has also been investigated as an optional antimicrobial treatment, and it follows the same principles of aPDT, however, the PS is excited by the application of the ultrasound (US) instead[13]. The SDT mechanism of action is not well established and researchers suggest that when the ultrassound is applied, it generates oscillated bubbles in the media that enlarge, collapse and implode, then, there is the production of bursts of lights that are able to activate the PS as the same way as in aPDT[13]. Some works have demonstrated the potential of SDT against bacteria and fungal suspensions, however, none of them achieved eradication against biofilms[14,15,16,17,18].

A new promising strategy to enhance the efficacy of the treatments is the association of both therapies (SDT and aPDT), called Sonophotodynamic Therapy (SPDT). The use of ultrasound in combination with the light improves the inactivation by disrupting the biofilm, enabling penetration of the sensitizer more deeply into the biofilm[19]. Alves et al. compared aPDT, SDT and SPDT, mediated by Photodithazine and Rose bengal, against *Candida albicans* biofilms, and authors observed that there was a synergism effect of the US in combination with aPDT (SPDT), where SPDT was more effective than aPDT or SDT[19]. Based on these promising results, the aim of this study was to evaluate the potential of SPDT, mediated by Curcumin, to inactivate *S. aureus* biofilm.

2. MATERIALS AND METHODS

2.1 Photosensitizer, light source and ultrasound device

The curcumin was used as photosensitizer. A stock solution of 16 mM was prepared in DMSO, then, this solution was diluted in sterile saline to the final concentration of 80 μM (keeping the final concentration of DMSO at 0,5%). The Technical Support Laboratory (LAT), from the São Carlos Institute of Physics (IFSC), São Paulo University (USP) developed a equipment with a blue LED device and ultrasound conjugated (Figure 1). The LED-based device (Figure 1, B) exhibits wavelength of 455 nm, it is composed of one blue LED (LXHL-PR09, Luxeon® III Emitter, Lumileds Lighting, San Jose, CA, USA) with intensity of 37 mW/cm^2. The ultrasound, coupled in the same device, was used at a frequency of 1 MHz, pulse repetition frequency of 100 Hz, 20% of duty cycle, and 3 W/cm² of intensity (Figure 1, A).

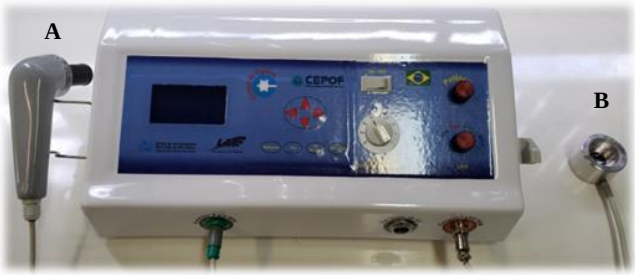

Figure 1. Ultrasound (A) and blue LED light device (B) developed by the Technical Support Laboratory, IFSC, USP.

2.2 Bacteria and biofilm formation

The methicilin-sensitive *Staphylococcus aureus* from American Type Culture Collection (ATCC), number 25923, was selected for this study. The bacteria that was storage at -20 ºC in tubes containing Tryptic Soy Broth (TSB) with 50% glycerol, was reactivated in Brain Heart Infusion (BHI) agar plates in incubator at 37 °C for 24 hours. After incubation, 5–10 colonies were resuspended in a tube with 10 mL of TSB and the bacteria was incubated at 37 °C for 16 hours. An aliquot of 500 μL of the suspension was diluted in 9.5 mL of fresh TSB and incubated until the mid-log phase of growth. The suspension was standardized at optical density (O.D.) of 0.2 arbitrary units (a.u.), determinated in spectrophotometer

(Varian Cary® 50 UV-Vis Spectrophotometer, Agilent, Santa Clara, California, United States), equivalent to 10^8 CFU/mL.

For biofilm formation, 1 mL of the bacteria standardized suspension was transferred to cell culture petri dishes and they were incubated at 37 °C in a shaker incubator (75 rpm) for 90 min (adhesion phase). After 90 min, the petri dishes were washed twice with phosphate-buffered saline (PBS) to remove non-adhered cells. Then, 1 mL of TSB was added to each petri dish. After incubation for 48 h in an orbital shaker (75 rpm) for biofilm formation, the suspension of each petri dish was removed and the biofilms were washed twice with PBS.

2.3 Treatments

After 48 h of biofilm formation, the samples were submitted to SDT, aPDT or SPDT. For SDT, biofilms were incubated with 2 mL of Cur at 80 μM, and the plates were incubated in the dark for 20 min. Afterwards, the US transducer was applied over the biofilms, at the frequency of 1 MHz, a power density of 3 W/cm², 20% of duty cycle and pulse frequency of 100 Hz for 32 min (SDT group). For aPDT, biofilms were incubated for 20 min with 2 mL of the sensitizer, then, the plate were irradiated with the blue LED light (70 J/cm²) (aPDT group). Finally, for SPDT, additional samples were incubated for 20 min with Cur and, then, both light and US were applied simultaneously using the same parameters as described previously. After treatments, the biofilm was detached by rubbing the pipette tip for 30 s on the bottom of the petri dish. To determine cell survival of the biofilms, aliquots of the contents of each sample were serially diluted 10-fold in sterile saline. Duplicate 25 μL aliquots were spread over the surfaces of BHI agar plates. All plates were aerobically incubated at 37 °C for 48 h. Then, the colony forming units (CFU/mL) were calculated. Extra biofilms were treated with the sensitizer (Cur group), US (US group) or LED light only (Light group), or no treatment (Control group). The treatments were performed in duplicate on three separated occasions.

2.4 Statistical analysis

The CFU/mL values were transformed into $\log_{10}$ and the homogeneity of variance and normality of the data were verified by the Levene and Shapiro–Wilk tests, respectively. Data was analyzed statistically by one-way analysis of variance (one-way ANOVA) and, for multiple comparisons, the post-hoc Tukey test was applied ($\alpha = 0.05$). These analyses were performed using a SPSS software package (IBM® SPSS® Statistics, version 20, Chicago, IL, USA).

3. RESULTS AND DISCUSSION

This study compared the aPDT, SDT and SPDT treatments, mediated by Cur, against *S. aureus* biofilm. It was observed that SPDT was more effective than aPDT and SDT. The association of US with aPDT (SPDT group) resulted in higher reductions of the biofilms, equivalent to 3.5 $\log_{10}$, while aPDT and SDT reduced 2.3 and 1.7 $\log_{10}$, respectively, in comparison with the control group ($p \leqslant 0.05$). There was no statistical difference between SDT and aPDT ($p=0.288$), and SPDT was statistically different from all groups ($p \leqslant 0.05$). The isolated application of Cur, light or US alone were similar to the untreated sample ($p=1.000$).

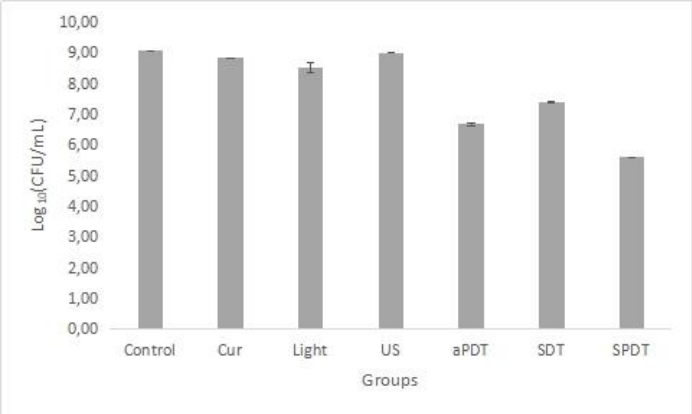

Figure 2. Mean values and standard deviation of Log_{10}(CFU/mL) of *S. aureus* biofilms treated with aPDT, SDT, SPDT mediated by Cur and control groups (control, Cur, light and US groups).

These results are in agreement with those found in the literature, where the use of US in combination with aPDT improves the efficacy of the treatment. Alves et al.[19], evaluated SDT, aPDT and SPDT, mediated by Photodithazine and Rose Bengal, against *C. albicans* biofilms. Authors observed that while individual aPDT or SDT had little impact on biofilms, combined aPDT/SDT significantly reduced the viability of *C. albicans* biofilms. The mechanisms that are involved in this synergism may be related to some US effects on the cells. According to Costley et al.[20], the US facilitates the dispersion of sensitizers through the biofilms, thus, improving efficacy of the treatment. Besides that, Bao et al. observed that the US increases the uptake of molecules through transient pores formed in the membrane, a process called sonoporation[21]. For this reason, exogenous particles, such as the photosensitizer, is able to penetrate into the cells through these pores. Another hypothesis is that the physical agitation of the solution originated by the US, causes circulation of the microorganisms and increases the exposure of them to the light.

4. CONCLUSION

In conclusion, the results demonstrated that SPDT is a promising strategy for the inactivation of *S. aureus* biofilms. A synergism was observed when the biofilms were treated with US and aPDT compared to aPDT and SDT alone. Authors suggest that the synergy may be related to the US effects on the cells that turn them more sensitized to the aPDT treatment.

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