

Antimicrobial susceptibility of photodynamic therapy (UVA/riboflavin) against *Staphylococcus aureus*

Suscetibilidade antimicrobiana da terapia fotodinâmica (UVA/riboflavina) contra *Staphylococcus aureus*

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ABSTRACT

Purpose: To assess *S. aureus* in vitro viability after the exposure to ultraviolet light A (UVA) and riboflavin (B2).

Methods: Samples of *S. aureus* in 96 well plates (in triplicate) were exposed to riboflavin (B2) and ultraviolet light A (365 nm wavelength) at a power density of 3 mW/cm², 8 mm spot diameter, for 30 minutes. Control groups were prepared as well in triplicate: blank control, ultraviolet light A only, riboflavin only and dead bacteria Control. The bacterial viability was measured using fluorescent microscopy. In order to investigate the occurrence of "viable but non-culturable" microorganisms after treatment, the cell viability was also investigated by plate culture procedure onto a broth medium. Statistical analysis was performed using the triplicate values from each experimental condition.

Results: No difference was observed among the treatment group and the control samples ($p=1$).

Conclusion: The combination of riboflavin 0.1% and ultraviolet light A at 365 nm did not exhibit antimicrobial activity against oxacillin susceptible *S. aureus*.

Keywords: Keratitis; Riboflavin; Corneal ulcer; *Staphylococcus aureus*; Ultraviolet rays; Cross-linking reagents

RESUMO

Objetivo: Avaliar a viabilidade celular de *S. aureus* in vitro após a exposição de riboflavina (B2) e luz ultravioleta A (UVA).

Métodos: Amostras de *S. aureus* colocadas em uma placa de 96 poços (em triplicata) foram expostas a riboflavina 0,1% (B2) e luz ultravioleta (comprimento de onda de 365 nm) poder de 3 mW/cm², 8 mm de diâmetro, por 30 minutos. Grupos controles foram também preparados em triplicata: controle branco, somente luz ultravioleta A, somente riboflavina e controle morto. A viabilidade bacteriana foi analisada usando microscópio de fluorescência. Para investigar a ocorrência de micro-organismos "viáveis porém não cultiváveis" a viabilidade celular foi avaliada utilizando-se placas de meio de cultivo bacteriano. Análise estatística foi realizada utilizando-se os valores obtidos em triplicata de cada grupo experimental.

Resultados: Nenhuma diferença foi observada entre o grupo tratamento e os grupos controle ($p=1$).

Conclusão: A combinação riboflavina 0,1% e luz ultravioleta 365 nm de comprimento de onda não demonstrou atividade antimicrobiana contra *S. aureus* oxacilina sensível.

Descritores: Ceratite; Riboflavina; Úlcera da córnea; *Staphylococcus aureus*; Raios ultravioleta; Reagentes para ligações cruzadas

INTRODUCTION

Microbial keratitis is a sight-threatening disease due to injury or trauma of corneal surface, and predisposing factors include poor contact lens hygiene⁽¹⁾. Successful treatment requires prompt characterization of the causative agent and application of specific chemotherapeutic therapy^(2,3). Since the introduction of sulfonamides and penicillin in the treatment of bacterial keratitis, an evolution in therapy has been observed^(3,4). However, virulence factors have decreased effectiveness of antibiotic monotherapy. One possible reason to explain the occurrence of antibiotic-resistant bacteria could be the selection of clones related with the increasing resistance factors faced on the development of newest and broad range aggressive therapeutic profiles. In addition, delay in the selection of appropriate antibiotic patterns may represent limiting factors of therapy and/or could provide development of bacterial resistance mechanisms^(5,6).

Bacterial keratitis due to *Staphylococcus aureus* is an increasingly common ocular infection⁽⁷⁾. Virulence factors of bacterium involve

expression of exotoxins and subversion of neutrophil-mediated host defense system⁽⁸⁾, which can result in severe inflammation, pain, corneal perforation, scarring, and loss of visual acuity. In general, staphylococcal resistance apparatus against antimicrobial agents include both intrinsic physical and genetic factors, i.e. cell-wall thickness due to peptidoglycan synthesis and gene expression on a specific staphylococcal cassette chromosome, respectively^(9,10).

The occurrence of antibiotic-resistant bacteria, with emphasis in staphylococcal infection, has prompted ophthalmologists to study the antimicrobial activity of additional biological, chemical and physical sources as adjunctive or alternative therapies for bacterial keratitis. Given that, UV-radiation can cause biosynthesis failures leading to cell death⁽¹¹⁾, the application of long-wavelength ultraviolet light (UVA) associated with vitamin B2 (riboflavin), as a photosensitizer, can emerge as an alternative tool for inactivation of bacterial pathogens. Such therapy, has been proposed for inactivation or reduction of the amount of bacterial pathogens from blood components and corneal tissue^(12,13).

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In order to provide information concerning a potential therapeutic effect on Gram-positive bacterial keratitis, the antimicrobial effect of UVA light in association with B2 against *S. aureus* strain was evaluated.

METHODS

STAPHYLOCOCCUS AUREUS STRAIN

All standard reference strains assayed by antimicrobial susceptibility tests *in vitro* were derived from *S. aureus* (SA) ATCC 29213. The bacterium was grown for 24h at 37°C in Nutrient broth medium (Difco, Franklin Lakes, NJ, USA) and the turbidity of cell concentration was adjusted to match a no. 0.5 McFarland optical density standard. The bacterial solution used in all experiments *in vitro* was standardized at concentration of approximately 10⁸ colony forming units (CFU)/ml.

IN VITRO ASSAYS

In order to provide comparative parameters of the antibacterial effect of UVA light and riboflavin in *S. aureus*, four control groups were performed as showed in table 1. Each control group was assayed in triplicate.

In the experimental group, 40 µl of bacterial solution was homogenized with B2 solution 0.1% and placed into each well of a sterile 96-well microplate lid (Corning Life Science, Lowell, MA, USA). Each well of the microplate lid has internal diameter of 7.85 mm, thus ensuring that the entire area can be exposed to UV light spot diameter of 8 mm, in a thin layer. Homogenized solution of *S. aureus* and riboflavin was exposed to UVA light radiation provided by a dedicated source (Opto Xlink, Opto, São Carlos, SP, Brazil) delivering wavelengths of 365 nm and irradiance of 3 mW/cm², for 30 min. After the UVA light exposure the homogenized sample was recovered from each well and the bacterial viability was assayed by using the LIVE/DEAD BacLight bacterial viability staining kit (Molecular Probes Inc., Eugene, OR, USA). Briefly, 5 µl of bacteria/ riboflavin irradiated mixture was homogenized with two fluorescent nucleic acid stains (SYTO9 and propidium iodide). The solution was placed onto a glass slide, covered with a coverslip and sealed. Bacteria were visualized under an Olympus IX81 inverted microscope (Olympus America Inc. Center Valley, PA, USA) equipped with filter packs GFP3035B and Texas Red 4040B (Semrock, Inc. Rochester, NJ, USA). The fluorescent digital images were captured by a Hamamatsu Photonics C9100-02 EMCCD camera (Hamamatsu Photonics, Japan), at a magnification of 40x. The red and green fluorescence was measured using the Imaris software (Bitplane Inc, South Windsor, CT, USA).

The leftover mixture of bacteria/riboflavin, was diluted in sterile saline solution (0.85%) to reach a final volume of 100 µl. After homo-

genization this suspension was inoculated onto a blood-sheep agar plate (BD Franklin Lakes, NJ, USA) by a spread plate technique. After incubation at 37°C for 24h in an ambient-air incubator, the bacterial growth was evaluated qualitatively; assuming that each viable bacterium in suspension should form an individual colony on the culture medium. Both microscopic and plate-culture procedures in the experimental group were done in triplicate.

STATISTICAL ANALYSES

For statistical analyses, the mean value of all three measurements was calculated and compared among groups with the Kruskal-Wallis test. Bonferroni method was used to correct for multiple comparisons. A p-value <0.05 was considered statistically significant. All analyses were done with Stata v. 10 (College Park, Texas, USA).

RESULTS

Antibacterial properties of UVA light and riboflavin were assayed by both microscopic and plate culture methods. The SYTO® 9 green-fluorescent nucleic acid stain labels all bacteria; those with intact membranes and those with damaged membranes. On the other hand the propidium iodide labels in red only bacteria with damaged membrane, causing a reduction in the green stain fluorescence when both dyes are present. The only group who presented cells stained in red, demonstrating damaged cells membrane, were the samples treated with 70% isopropyl alcohol, with 100% red fluorescence. (Figure 1) All the other groups (live control, only UVA, riboflavin alone (B2) and UVA-B2) revealed viable cells stained in green fluorescence. The mean green fluorescence percentage was 99.8%, 99.8%, 99.6% and 99.7%, respectively for the blank control, only UVA light, only riboflavin and UVA-B2 respectively (Figure 1). There was no statistical difference in the amount of green fluorescence among the groups.

The results shown in figure 2 reveal neither bactericidal nor bacteriostatic activities in control groups 2 and 3, which evaluated the individual effect of UVA and B2 in the cell viability of *S. aureus*, respectively. In addition, control group 4, which represented the dead control, showed bactericidal effect for the most of the cells and bacteriostatic effect for those who formed individual colonies (Figures 2, A4 and B4). In the experimental group, which evaluated the combined antibacterial effect of UVA light and riboflavin, the *S. aureus* proved to be resistant to this physical-chemical technology under the parameters used in our experiments. Figures 2-A5 and 2-B5 show a countless amount of viable bacteria. This corroborates the results obtained from the individual application of UVA and riboflavin, which was demonstrated neither bacteriostatic nor bactericidal effect in *S. aureus*.

Table 1. Control groups used as comparative parameters in the *in vitro* assays

Control groups	<i>In vitro</i> assays
Blank control (control 1)	Bacterial cells recovered from each microplate well at the end of experiment without exposure to UV lights neither riboflavin solution.
UVA light (control 2)	Bacterial cells recovered from each microplate well previously submitted to 30 minutes of UVA exposure without riboflavin instillation.
Riboflavin (control 3)	Riboflavin-5-phosphate (Sigma-Aldrich, St. Louis, MO) was added to the cell solution at final concentration of 0.1% and the bacterial viability was assessed 30 min after contact, without UVA exposure.
Dead control (control 4)	Bacterial cells were treated with 70% isopropyl alcohol for 1 hour.

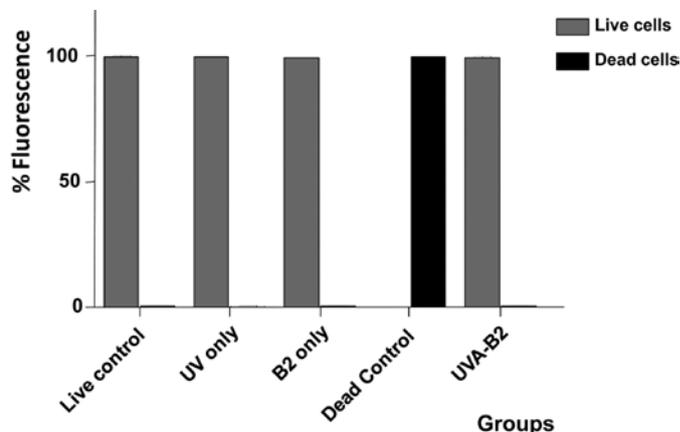


Figure 1. Percentage of red and green fluorescence in each group.

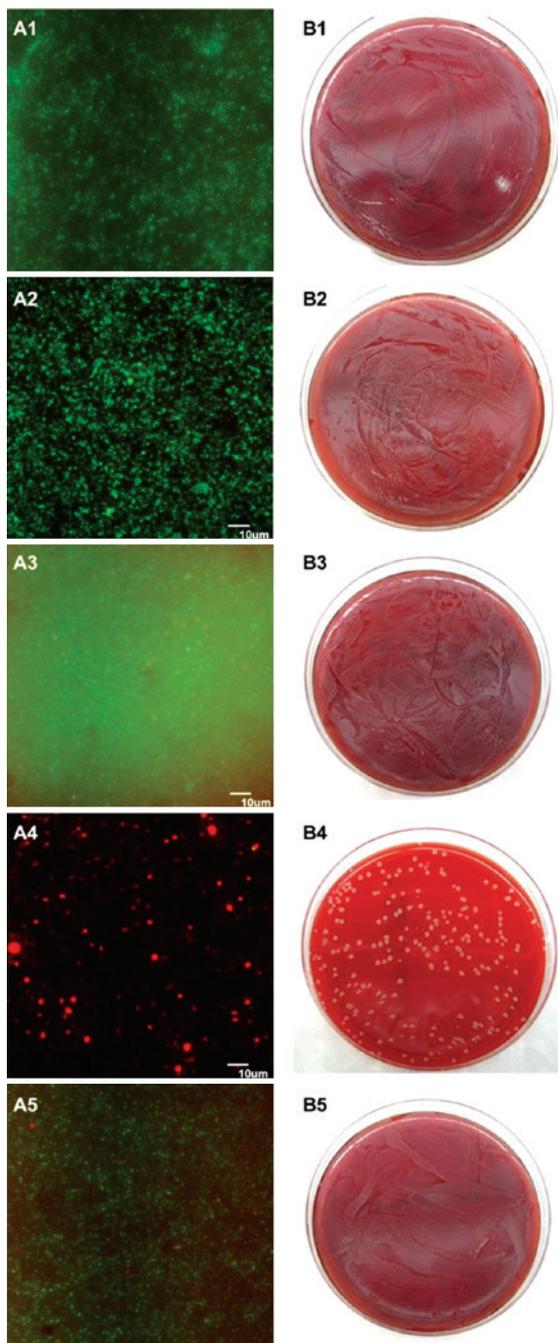


Figure 2. A) Fluorescence pictures of *S. aureus* stained by LIVE/DEAD® BacLight Bacterial Viability. Live cells stained by SYTO® 9 green-fluorescent and the dead cells stained by propidium iodide, magnification of 40x; B) Qualitative bacterial growth in blood agar, 24 hours after UVA-B2 exposure. 1: alive control; 2: only UVA; 3: only riboflavin 4: dead control; 5: UVA-B2. All samples but B4 showing countless amount of colonies. B4 shows bactericidal effect for the most of the cells and bacteriostatic effect for those who formed individual colonies.

DISCUSSION

The rationale for use of UVA light associated with vitamin B2 for infectious keratitis treatment is based mainly on the riboflavin-based pathogen reduction technology (PRT) used in the microbial disinfection process of blood products^(14,15). However, the UVA-B2 procedure designed to therapeutic application on keratoconus, which was evaluated in this study, uses UVA light wavelength of 370 ± 5 nm and

total irradiance dose of 3.4 J based on safety studies⁽¹⁶⁾. In comparison the riboflavin-based pathogen reduction technology (PRT) delivers higher total energy (6.2 J) as well as a shorter wavelength (265 to 370 nm)⁽¹⁷⁾, which could cause more damage to the cells and DNA of pathogen, providing effective action in the bacterial death. Basically, the infectivity of pathogens is reduced by three complementary procedures: (1) the direct damage of nucleic acids of the pathogens by the UVA light, (2) the damage of proteins and membranes of microorganisms by reactive oxygen species generated when riboflavin absorbs light and interacts with dissolved oxygen in solution and (3) the damage of genetic machinery by the interaction of riboflavin with microbial nucleic acids^(18,19).

The 'gold-standard' method, which is proposed to determine the microbial viability, is the growth of colonies on a nutrient agar surface after a period of incubation under a specific temperature and atmospheric conditions rates⁽²⁰⁾. The plate count method is based on the premise that each viable bacterium can grow, divide and become a colony, via binary fission in a suitable growth medium. Thus, unlike bacterial counts by the method of direct microscopy in which all cells (dead and alive) are counted, the plate culture procedure allows qualitative and quantitative analysis of viable cells in a sample. However, in the experimental protocols involving nutrient deprivation, environmental stress or antimicrobial effect of biological, chemical and/or physical agents the cultivation method can fail to assess the cell viability if the target pathogen enters into the metabolic dormant stage of "viable but non-culturable" (VBNC)^(21,22). In general, bacteria in the VBNC state are not able to grow on the artificial culture media, but they are alive and capable of renewed metabolic activity⁽²⁰⁾. One of the approaches to determine the VBNC state is establishing the presence of an intact cytoplasmic membrane. For this reason, a methodological approach based on additional testing of fluorescent dye to study the bacterial viability was proposed in this study. The SYTO® 9 green-fluorescent nucleic acid stain labels all bacteria those with intact membrane and those with damaged membrane, while the propidium iodide labels in red only bacterial with damaged membrane, causing a reduction in the green stain fluorescence when both dyes are present⁽²³⁾.

In a previous study, Martins et al.⁽²⁴⁾ demonstrated no bacterial growth in a suitable media 24h after UVA-B2 exposure. However, a hypothesis that leads the cells to enter in a VBNC metabolic state could be the presence of free radicals⁽²¹⁾. Some authors⁽²⁵⁾ suggested that non-growing cells might produce free radicals on exposure to high nutrient-containing medium, which might prevent cell division and the origins of new colonies. The non bacterial growth after UVA-B2 exposure observed in that experimental study could be justified by the presence of production of singlet oxygen, and generation of hydrogen peroxide. Thus, those bacterial cells assayed under the UVA-B2 exposure might not be in fact dead, but instead in a VBNC state.

The main goal of the study was first observe the efficacy of the combination UVA-B2 as a bactericidal agent. We were able to demonstrate the bacterial viability only by using the fluorescent dyes as done in previous studies^(11,21). Thereafter, we did not consider to count the exact amount of colonies using the traditional dilution methods, instead, the objective of placing the leftover treated sample and the control groups onto a plate culture were merely illustrative, showing that the groups stained in green were able to grow in a plate culture, meanwhile the group stained mostly in red only a few cells were able to grow in the plate culture. Curve response was not performed because a safety protocol previous established for this technology was followed in order to avoid the occurrence of intra-ocular damages, mainly by the UVA light⁽¹⁶⁾. Instead, we have tried higher concentrations of riboflavin, but riboflavin 0.5% and 1% in the presence of the fluorescence dyes caused a fast bleaching which preclude high quality fluorescence microscopic data.

In conclusion, we were able to demonstrate a qualitative agreement concerning the lack of efficacy of photodynamic therapy

against *S. aureus*. However, the quantitative data concerning the agreement of antibacterial effect of components of photodynamic therapy was elusive due to staphylococcal resistance against both physical (UV light) and chemical (riboflavin) agents tested and, consequently, the occurrence of uncountable amount of bacteria into culture media. Finally, the current related that there was no bactericidal effect of UVA/B2 settings used in treatment of keratoconus, against oxacillin susceptible *S. aureus*.

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