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In Vitro Evaluation of Rose Bengal Photoactivated by Custom-Built Green Light-Emitting Diode Source for Bacteria and Rapidly Growing Mycobacteria Inhibition

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Purpose: In vitro evaluation of rose bengal (RB) photoactivated by our custom-built green light-emitting diode (LED) source for the growth inhibition of bacterial strains and rapidly growing mycobacterial (RGM) isolates in infectious keratitis.

Methods: Six corneal clinical bacteria isolates were included in this study: two Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* [MRSA] and *Staphylococcus epidermidis*), two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Serratia marcescens*), and two RGM (*Mycobacterium chelonae* and *Mycobacterium abscessus*). Microorganisms were cultured and incubated at specific conditions and prepared in suspensions to adjust their concentration to 10^4 cells/mL. Different treatments were conducted in triplicates: Group I, no treatment; Group II, treated with 0.1% rose bengal alone (exposed to dark for 30 minutes); Group III, exposed to custom green LED for 30 minutes (12.87 J/cm^2); and Group IV, treated with 0.1% rose bengal and exposed to custom green LED for 30 minutes. Agar plates were incubated at specific conditions and photographed after growth for pixel analyses.

Results: Complete growth inhibition of all bacteria and RGM was observed in Group IV. MRSA and *S. epidermidis* in Group II also showed complete growth inhibition.

Conclusions: The custom-built green LED presented good activity by photoactivating RB and inhibiting micro-organism growth. For the first time, we demonstrated the expressive growth inhibition effect of RB against *S. epidermidis*, RGM, and *S. marcescens*. Clinical treatment with RB may offer an alternate adjunct therapy for corneal surface infections.

Translational Relevance: Validating in vitro the custom-built green LED encourages the clinical application for the treatment of infectious keratitis.

Objetivo: Avaliação in vitro de rosa bengala (RB) fotoativada por nossa fonte de luz diodo emissor (LED) verde customizado na inibição do crescimento de bactérias e micobactérias de crescimento rápido (MCR) isolados de ceratite infecciosa (CI).

Métodos: Seis bactérias isoladas de CI foram incluídas neste estudo: duas bactérias Gram-positivas (BGP) (*Staphylococcus aureus* resistente à meticilina [MRSA] e *S. epidermidis*), duas bactérias Gram-negativas (BGN) *Pseudomonas aeruginosa* e *Serratia marcescens*) e duas MCR (*Mycobacterium chelonae* e *M. abscessus*). Micro-organismos foram cultivados e incubados em condições específicas e suspendidos para ajustar sua concentração a 10^4 células/mL. Diferentes tratamentos foram conduzidos em triplicata: Grupo I, sem tratamento; Grupo II, tratado apenas com RB 0,1% (exposto ao escuro por 30 min); Grupo III, exposto a LED verde por 30 min (12,87 J/cm^2); e Grupo IV, tratado

com RB a 0,1% e exposto a LED verde por 30 min. As placas de ágar foram incubadas em condições específicas e fotografadas após o crescimento para análise de pixels.

Resultados: Observou-se inibição completa do crescimento de todas as bactérias e MCR no Grupo IV. As BGP no Grupo II também apresentaram inibição completa do crescimento.

Conclusões: O LED verde apresentou boa atividade fotoativando a RB e inibindo o crescimento de micro-organismos. Pela primeira vez, demonstramos o efeito da RB contra *S. epidermidis*, MCR e *S. marcescens*. O tratamento clínico com RB pode oferecer uma terapia adjuvante alternativa para CI.

Relevância Translacional: A validação in vitro do LED verde customizado encoraja à aplicação clínica para o tratamento de CI.

Introduction

Microbial keratitis (MK) is one of the leading causes of corneal blindness worldwide and can lead to severe complications with secondary permanent vision loss[1]; additionally, a growing factor that may obscure the prognosis in such cases is the antibiotic resistance.[2] Photodynamic therapy (PDT) was first described for corneal collagen cross-linking (CXL) to treat keratoconus using riboflavin as a photosensitizing agent and ultraviolet-A (UV-A) irradiation[3]; later, PDT-CXL was introduced as an alternative treatment for corneal infections.[4] Despite several successful case reports,[5,6] PDT-CXL is generally considered to be ineffective against fungal[7] and parasitic[8] infections, and, as previously mentioned, bacteria resistance to standard medical treatment with topical antibiotics is emerging, and thus alternative[9] treatment methods need to be developed.

PDT involves the activation of a photosensitizing agent by light ranging. An excited photosensitizer reacts with ambient oxygen to produce a reactive oxygen species that reacts with the intracellular components and lead to cell inactivation and death.[10] In vitro studies on rose bengal photodynamic antimicrobial therapy (RB-PDAT) have demonstrated its effectiveness against several microbial species,[11–13] including *Pseudomonas aeruginosa* and *Fusarium solani*. Moreover, RB-PDAT produced better results compared with riboflavin PDT against multidrug-resistant bacteria,[14] yeast,[15] filamentous fungi,[16] and parasites.[8] Nowadays, most experiments on the in vitro and in vivo activity of RB-PDAT are performed using a customized green light-emitting diode (LED) from the Ophthalmic Biophysics Center of the University of Miami[15] and have showed promising results. Despite multiple studies evaluating the effectiveness of RB-PDAT, a lot of its details remain unclear, including its activity against mycobacteria.

The usual etiological agent responsible for MK varies with geographical regions.[17] Among all Gram-positive and Gram-negative bacteria, *Staphylococcus aureus* and *P. aeruginosa* are the main bacteria that cause infectious keratitis,[18] followed by *Staphylococcus epidermidis* and *Serratia* spp.[19,20] Because there is no commercially available green light source to be used for this procedure, it was necessary to validate a custom-built source in vitro and clinically and compare it with the prototype at the Ophthalmic Biophysics Center of the University of Miami, built for this purpose. Therefore this study aimed to assess the activity of our custom-built green LED as a light source for bacteria already tested for RB-PDAT in vitro[12,14] and to evaluate, for the first time, the RB-PDAT activity for the in vitro growth inhibition of the most prevalent bacterial isolates in infectious keratitis and rapidly growing mycobacteria.

Methods

Light Source

A custom-built green LED was fabricated at the Institute of Physics, University of São Paulo, São Carlos–Brazil, with one green 3W LED placed in an aluminum heatsink (Fig. 1A) based on the prototype built at the Ophthalmic Biophysics Center of the University of Miami.[15] The green LED (Cromatek, São Paulo, Brazil) had a central wavelength at 515.5 nm (±20 nm), measured using a USB 2000 spectrometer (Ocean Optics, Orlando, FL, USA). Irradiance was measured using a FieldMaxII-TOP power meter (Coherent, Santa Clara, CA, USA) that produced 7.15 mW when the LED was placed 3 cm away from the plate (Fig. 1B).

Isolate Inoculum

Experiments were performed at the ocular microbiology laboratory at the Ophthalmology Department of Escola Paulista de Medicina–Universidade Federal

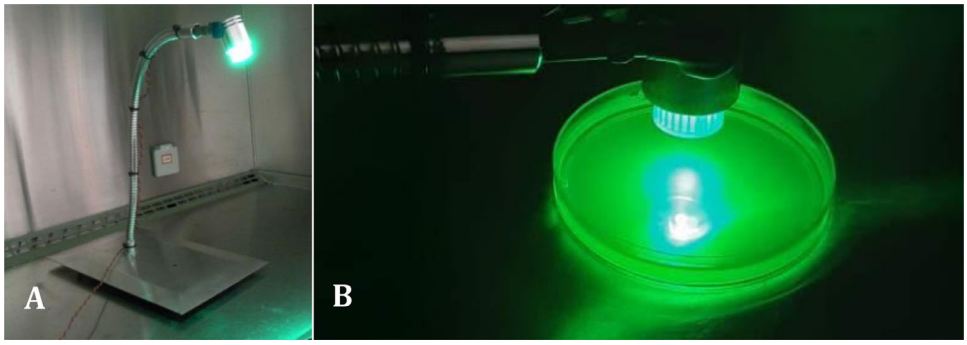

Figure 1. (**A**) Custom-built green LED. (**B**) The LED placed 3 cm away from the plate.

de São Paulo following the protocol by Arboleda et al., with some modifications.[15] Six bacterial isolates were obtained from the corneal scrapings of patients diagnosed with bacterial keratitis at the São Paulo Hospital, Federal University of São Paulo; these included two Gram-positive bacteria, two Gram-negative bacteria, and two rapidly growing mycobacteria. The Gram-positive bacteria included methicillin-resistant *S. aureus* (MRSA) that was also resistant to gentamicin, tobramycin, azithromycin, ciprofloxacin, and moxifloxacin through the microdilution method and *S. epidermidis* that was susceptible to oxacillin, gentamicin, tobramycin, azithromycin but resistant to ciprofloxacin and moxifloxacin through the microdilution method. The Gram-negative bacteria included *P. aeruginosa* resistant to gentamicin, tobramycin, amikacin, ciprofloxacin, and moxifloxacin through the microdilution method and *Serratia marcescens* susceptible to gentamicin, tobramycin, and amikacin but resistant to ciprofloxacin and moxifloxacin through the microdilution method. The mycobacteria included *Mycobacterium chelonae* susceptible to tobramycin, amikacin, and linezolid but resistant to ciprofloxacin and moxifloxacin through the microdilution method and *Mycobacterium abscessus* susceptible to amikacin and linezolid but resistant to tobramycin, ciprofloxacin, and moxifloxacin by microdilution method. All isolates were confirmed using traditional and phenotypic microbiological techniques.

Gram-positive and Gram-negative bacteria were recovered on nutrient agar (Kasvi, São José dos Pinhais, Paraná, Brazil) plates for three days at 37°C, and mycobacteria were recovered on Difco Middlebrook 7H10 Agar (BD Becton Dickinson, Franklin Lakes, NJ, USA) plates for five days at 30°C. A 0.5-McFarland (1.5×10^8 colony-forming unit per mL [CFU/mL]) suspension of each strain was prepared in ultra-pure water (Sigma-Aldrich, Co. Ltd, St. Louis, MO, USA) using Oxoid Turbidimeter (Thermo Scientific, Waltham, MA, USA), followed by 10-fold dilutions prepared in ultra-pure water to reach a final concentration of 10^4 CFU/mL.

Treatment Protocol

Each bacterial isolate was distributed into four groups and each group was subjected to different treatments in triplicates: Group I (no treatment), 1 mL of the inoculum (10^4 CFU/mL) was added to the agar plate; Group II, 1 mL of the inoculum (10^4 CFU/mL) was treated with 0.1% rose bengal eye drop (0.9mL 10^5 suspension and 0.1 mL of 1% rose bengal eye drop) (EYE/PHARMA São Paulo, São Paulo, Brazil), added to the agar plate, and exposed to dark for 30 minutes; Group III, 1 mL of the inoculum (10^4 CFU/mL) was added to the agar plate and exposed to green LED light for 30 minutes (12.87 J/cm^2); and Group IV, 1 mL of the inoculum (10^4 CFU/mL) was treated with 0.1% rose bengal, added to the agar plate, and exposed to green LED light for 30 minutes (12.87 J/cm^2). Using a digital infrared thermometer (Lasertec, São Paulo, São Paulo, Brazil), the temperature of those plates exposed to the green LED light were measured, before and right after exposure. Gram-positive and Gram-negative bacteria were plated on nutrient agar and incubated for three days at 37°C. Mycobacteria were incubated on Middlebrook 7H10 Agar plates for five days at 30°C. This study was approved by the Institutional Ethics Committee of the Federal University of São Paulo (UNIFESP IRB approval: 1.423.852).

Inhibition Growth Assessment

Images of the agar plates were taken after incubation using a cellphone camera (Samsung A30, 2019; Samsung, Seoul, South Korea). For the inhibition analysis, the agar plate images were taken in two distinct time points: (i) at the time of treatment (image

I_I) and (ii) after incubation (image I_F). The agar plate diameter was 90 mm, and the region of interest (ROI) (green light irradiation area) was determined as a central circular region with a diameter of 40 mm. Image analysis involved decomposing the original image in its color channels R (red), G (green), and B (blue) because it is more appropriate to perform the processing steps using the G channel because of the high contrast. Thus the green component of the image can be represented by a grayscale image, with intensity values ranging from 0 (black) to 255 (white). The Hough Circle Transform was then used to automatically determine both the agar plate region and ROI for inhibition analysis. To evaluate the growth of micro-organisms on the plates, a pixel-based analysis was performed. Therefore, for each set of I_I and I_F images, the average pixel intensity of the ROI of I_I was obtained to determine the threshold value T, which characterizes the expected pixel intensity for agar plates without growth. Based on the T value, a new image, I_T, was created using Equation 1 below, where I_F (x_r,y_r) represents the pixels of the ROI of image I_F.

$$I_T(x,y) = \begin{cases} 0 & \text{if } I_F(x_r,y_r) \le T \\ 255 & \text{if } I_F(x_r,y_r) \ge T \end{cases} \quad (1)$$

Thus the resulting image I_T comprised a binary image, representing only the ROI, with the micro-organisms represented as white pixels and regions with no growth as black pixels. For each micro-organism evaluated, considering I_T, a measure of growth g was obtained based on the calculated ratio between the number of white pixels and the total amount of pixels in images. This value represents the absolute proportion of micro-organism growth in treated regions. A relative analysis was also performed to compare the growth in the control, g_c, with that in other treatments, g_t. The inhibition coefficient was calculated using the Equation 2 shown below:

$$IC = 1 - \frac{g_t}{g_c} \quad (2)$$

Results

Images collected after bacterial incubation are presented in Figure 2. For each plate, a central zone corresponding to the diameter of the irradiation source (40 mm) was selected for measurement. Results showed substantial growth inhibition after treatment with 0.1% rose bengal and exposure to green LED light for 30 minutes (Group IV) in all six isolates: MRSA (99.79%), *S. epidermidis* (100%), *P. aeruginosa* (97.85%), *Serratia* spp. (99.9%), *M. chelonae* (99.85%), and *M. abscessus* (91.93%) (Table). MRSA and *S. epidermidis* also showed substantial growth inhibition (99.78% and 100%, respectively) after treatment with 0.1% rose bengal and incubation in the dark for 30 minutes (Group II), whereas only slightly inhibition was shown by *P. aeruginosa* (18.72%), *M. chelonae* (17.24%), and *M. abscessus* (1.04%) (Table). After exposure to green LED light for 30 minutes (Group III), uniform growth was observed on the entire surface of agar plates for MRSA (11.74%), *P. aeruginosa* (15.60%), and *M. chelonae* (3.27%), and growth inhibition was limited when present. The temperature of the agar plate after a full 12.87 J/cm^2 exposure remain constant.

Discussion

Our findings support the hypothesis that RB-PDAT can effectively inhibit bacterial growth. Our custom-built green LED, which is similar to that at the Ophthalmic Biophysics Center at the University of Miami, worked as expected by photoactivating 0.1% of rose bengal and inhibiting bacterial growth. All six bacteria species exhibited complete growth inhibition after treatment with 0.1% rose bengal photoactivated by custom-built green LED (515.5 nm). Rose bengal alone exposed to dark conditions was also effective against MRSA and *S. epidermidis*. On green LED treatment only, growth inhibition was very limited when present. The reason is unknown, but we may state that it was not due to heat because the temperature remained constant.

To the best of our knowledge, this is the first study that examined the effectiveness of RB-PDAT in the growth inhibition of rapidly growing mycobacteria. Shih et al.[21] evaluated the antimicrobial effects of methylene blue (MB)–mediated PDT on *Mycobacterium fortuitum* keratitis in vitro and in an animal model experiment, indicating that MB-PDT is a potential alternative treatment for nontuberculous mycobacterial corneal infections. The standard treatment for infectious mycobacterial keratitis is usually challenging as its causative agent can be resistant to conventional antituberculous agents and can exhibit varied susceptibility to other available antibiotics[22] Previous studies have shown that riboflavin PDT is an effective adjunct therapy for mycobacterial keratitis[23,24]; our results suggest that RB-PDAT is another adjuvant therapeutic option.

Rose bengal alone shows antibacterial activity, which increases when photoactivated by green light.[25]

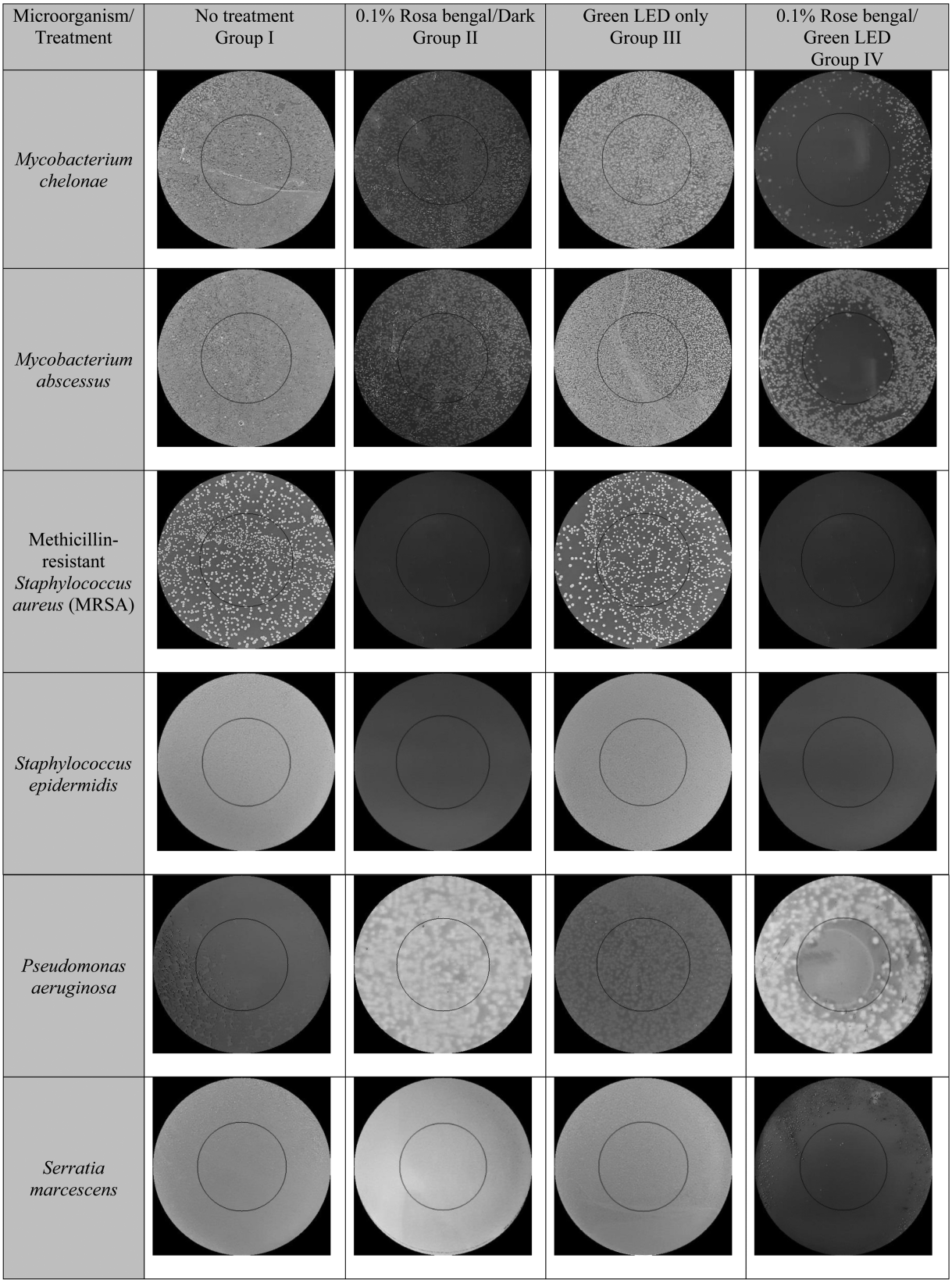

Figure 2. Assessment of RB-PDAT for the growth inhibition of bacterial keratitis isolates. For each plate, a central zone was selected corresponding to the diameter of the irradiation source (40 mm). Growth inhibition in 0.1% rose bengal/green light treatment (Group IV) for all six isolates (in the central zone for *M. chelonae*, *M. abscessus, P. aeruginosa*, *S. marcescens* and whole plate of MRSA and *S. epidermidis*) and 0.1% rose bengal/dark (Group II) for MRSA and *S. epidermidis (*whole plate).

Table. Percentage of Growth Inhibition of Bacterial Isolates According to Treatment

Microorganism/Treatment	No Treatment Group I	0.1% Rose Bengal/Dark Group II	Green LED Only Group III	0.1% Rose Bengal/Green LED Group IV
M. chelonae	0.00%	17.24%	3.27%	99.85%
M. abscessus	0.00%	1.04%	0.00%	91.93%
S. aureus (MRSA)	0.00%	99.78%	11.14%	99.79%
S. epidermidis	0.00%	100.00%	0.00%	100.00%
P. aeruginosa	0.00%	18.72%	15.60%	97.85%
S. marcescens	0.00%	0.00%	0.00%	99.90%

Halili et al.[14] examined the effect of two different concentrations of rose bengal (0.1% and 0.03%) on the growth inhibition of MRSA strains under dark and after exposure to green light (5.4 J/cm^2). Both concentrations inhibited bacterial growth after green light exposure; however, under dark conditions, only rose bengal at 0.1% was able to inhibit bacterial growth.[14] Thus it was expected that MRSA would be inhibited in both groups with 0.1% rose bengal (Group II and IV) in our study; however, although the results for *S. epidermidis* confirmed the same inhibition profile, it is uncertain whether another *Staphylococcus* spp. would have the same response.

Most cases with *P. aeruginosa* keratitis have good outcomes when appropriate antibiotic treatment was administered in the early stages. However, the increasing prevalence of multidrug-resistant *P. aeruginosa* emphasizes the need to develop alternative treatments. Although a previous study demonstrated that RB-PDAT inhibits *P. aeruginosa*,[12] we aimed to test the effectiveness of our custom-built green LED and compare it with published results. Because the most prevalent Gram-negative bacterial isolates involved in keratitis are *P. aeruginosa* and *Serratia* spp., *S. marcescens* was included in this study. As per the results, its growth was also inhibited.

RB-PDAT has also been tested on other pathogens in vitro, such as *Aspergillus* spp., *Candida* spp., *Fusarium* spp., and *Achanthamoeba* spp., with good results.[11,13,15,16] Identifying the effectiveness of RB-PDAT against micro-organisms increases the confidence in using it in a clinical setting. In 2016, Zhu et al.[26] proved in an animal model, that RB-PDAT is a safe, rapid, and effective treatment for MK, with no evidence of retinal or iris damage even with higher energies of up to 150 J/cm^3. Amescua et al.[11] introduced the use of RB-PDAT for the treatment of infectious keratitis in humans with a successful clinical case after only two sessions of RB-PDAT and with no recurrence of *Fusarium keratoplasticum* infection or any adverse effects. In our study, we applied higher energy (12.87 J/cm^2) than the one used by Amescua et al.[11] (5.4 J/cm^2), yet the safety was maintained in an animal model.[26] Thereafter, scientists have attempted to highlight the clinical efficiency and safety of RB-PDAT for the treatment of infectious MK caused by bacteria and parasites, even in an animal model.[13,27] More recently, new proposals for RB-PDAT methodology and treatment have been presented.[23,28] Martinez et al.[29] demonstrated the safety of RB-PDAT using 0.1% RB, with no clinical and histopathological differences found between the treated and control groups.

The results presented in this study demonstrate the in vitro activity of RB-PDAT against the clinical isolates of *M. abscessus*, *M. chelonae*, *S. marcescens*, and *S. epidermidis* for the first time. Our custom-built green LED reproduced the results that were previously reported for *P. aeruginosa* and MRSA using the green LED at the Ophthalmic Biophysics Center at the University of Miami. This suggests that RB-PDAT is a possible adjunct therapy to treat bacterial corneal infections. Further studies are warranted to gather more information about its antifungal and antiparasitic activity, toxicity, and clinical efficacy.

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