BACTERICIDAL EFFECTS OF LOW-IRRADIANCE LOW LEVEL LIGHT THERAPY ON METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS IN VITRO*

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ABSTRACT

Low Level Light Therapy (LLLT) within the visible blue spectrum (400–470 nanometers) is a well-documented therapeutic alternative to combat multidrug resistant organism infections through the generation of reactive oxygen species (ROS). However, one shortcoming of LLLT is that many studies deliver therapy through high powered lasers and lamps. High powered light sources not only require specialized staff to operate, but they also deliver the total light dose (fluence) at an exceptionally high intensity, or irradiance, which could consequently deplete the oxygen supplies required to promote LLLT's bactericidal properties. To overcome these faults, low-irradiance LLLT, or delivering the same total fluence of LLLT over an extended period of time with decreased irradiance was evaluated *in vitro*. To further explore this alternative approach, the bactericidal effects of low-irradiance (10.44 mW/cm^2) LLLT using wavelengths of 405-nm, 422-nm and 470-nm were studied on methicillin-resistant *Staphylococcus aureus* (MRSA) cultures. Among these wavelengths, it was determined that 405-nm LLLT provided the most effective reduction of bacterial load at the lowest total fluence (75 J/cm^2) (94.50% reduction). The bactericidal effects of 405-nm low-irradiance LLLT were then further studied by treating MRSA cultures to 75 J/cm^2 LLLT while using irradiances of 5.22 mW/cm^2 and 3.48 mW/cm^2. It was concluded that there was a greater reduction of MRSA bacterial load when samples were exposed to irradiances of 5.22 mW/cm^2 (95.71% reduction) and 3.48 mW/cm^2 (99.63% reduction). This study validates the bactericidal properties of low-irradiance LLLT on MRSA, and subsequent studies should be completed to optimize its full therapeutic potential.

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KEYWORDS

- Photoinactivation
- bactericidal
- *MRSA*
- Low Level Light Therapy

INTRODUCTION

The plight of antibiotic resistance, partially due to the medical community's overreliance of antibiotics, has led to efforts by healthcare systems to determine alternative strategies for antimicrobial treatments. In the United States, there are more than 2 million cases of hospital-acquired infections (HAIs) annually, resulting in annual costs of $20 billion and nearly 100,000 deaths (16,26). Methicillin-resistant *Staphylococcus aureus*

(*MRSA*) is one of the prominent pathogens involved with both HAIs and community acquired infections (CAIs). The prevalence of an individual within the intensive care unit who develops either a HAI or CAI due to *MRSA* are greater than 55% and 59%, respectively (10). *MRSA* has been able to ascend to its present status as a predominant infective agent due to its ability to develop high levels of resistance to several classes of antibiotics through different pathways, including mutation, conjugation, transduction, and transformation (2).

There are two distinct sources of *MRSA*: (1) hospital-associated *MRSA* (HA-*MRSA*), which are limited to clinical settings, and (2) community-associated *MRSA* (CA-*MRSA*), which are found in a multitude of environments and settings, including beaches, computer keyboards, schools, gyms, athletic fields, and locker rooms. (5-7). *MRSA*, an opportunistic pathogen, is not only responsible for localized trauma or postoperative infections, but can also be life-threatening, causing bacteremia, endocarditis, sepsis or toxic-shock syndrome (11,13,17). Research also suggests *MRSA* is one of the primary pathogens responsible for failure of surgical implants or prosthetics due to biofilm formation at the soft-tissue-implant interface, which compromises patients' activities of daily living (ADLs), as well as causes re-implantation to eliminate the bacterial load (28).

A long-term solution to better manage multidrug-resistant organism infections is paramount. Low Level Light Therapy (LLLT), also referred to as "biostimulation," is the process of illuminating tissues with a precise wavelength of light over a specific period of time. More specifically, the use of high energy (lower wavelength) waves in the ultraviolet (UV) and visible blue spectra for LLLT have shown more consistent

results when it comes to bactericidal effects and reduction of bioburden. The capabilities of UV (100-400nm) LLLT to sterilize wound surfaces has been well-documented (3,8-9,15). Dai *et al.* investigated the potential of prophylactic UV (200-280nm) light treatment for infections developing in superficial cutaneous mouse wounds contaminated with both *MRSA* and *Pseudomonas aeruginosa* (9). For both bacterial infections, UV LLLT significantly reduced the bacterial burden in comparison to untreated wounds, while also increasing the survival rate of *P. aeruginosa* infected mice (58%) and wound-healing rate of *MRSA* infected mice (31%) (9). However, a limitation to UV LLLT is that it is toxic and carcinogenic in tissues with extended exposure (3,8-9,15).

In order to overcome the collateral damage associated with UV LLLT, the use of wavelengths in the visible blue spectrum (400-470-nm) have been proven to be efficacious for both their bactericidal and wound healing capabilities *in vitro* and *in vivo* (1,10,19-20,23,27). While UV LLLT's bactericidal properties are induced through DNA damage, the mechanism of visible blue light involves the photoexcitation of endogenous porphyrins within bacterial species, which is much less detrimental to healthy mammalian cells (1,10,19-20,23, 27). Subsequently, this excitation initiates the formation of reactive oxygen species (ROS), which are toxic to bacterial cells and biofilms, without compromising the DNA of adjacent cells. Maclean *et al.* investigated the bactericidal effects of visible (405-nm) light using a high-intensity xenon lamp, and illustrated that the 405-nm array had a phototoxic effect on a variety of bacteria that are highly prevalent in CAIs and HAIs, including Gram-positive bacteria: *MRSA, Staphylococcus epidermidis, Streptococcus pyogenes, Clostridium perfringens,* and

Gram-negative bacteria: *Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris* and *Klebsiella pneumonia* (19).

The first underlying objective of this study was to compare the bactericidal efficacy of LLLT on cultures of *MRSA* using three specific wavelengths (405-nm, 422-nm and 470-nm) within the visible blue spectrum. Additionally, the objective of this study was to evaluate the efficacy of low-irradiance LLLT, or the concept of delivering the same total light dose, or fluence, of LLLT at a lower intensity, or irradiance, over an extended period of time. This concept contradicts standard LLLT where therapy is administered at a considerably high irradiance (>40 mW/cm^2) and delivered over a period of seconds to 15 minutes (10, 14).

Based on these objectives, this *in vitro* study was conducted in two separate phases. In phase 1, the bactericidal properties of 405-nm, 422-nm and 470-nm LLLT were evaluated to determine which wavelength provided the greatest bacterial load reduction at the lowest possible fluence. MRSA samples were exposed to these three wavelengths at an irradiance of 10.44 mW/cm^2, which is substantially lower than precedent studies (10, 14). After determining the optimal wavelength and the precise fluence, phase 2 was conducted by evaluating the efficacy of low-irradiance LLLT at the optimal wavelength, but at irradiances of 5.22mW/cm^2 and 3.48 mW/cm^2, and durations of 4 and 6 hours. Given the recent advancement of implantable and portable LLLT devices (4, 25), the process of administering LLLT over a period of multiple hours has the potential to be a cost-effective yet clinically feasible alternative to conventional LLLT.

Despite the fact LLLT's bactericidal effects have been well documented within all wavelengths of the visible blue spectrum, it is hypothesized that 405-nm LLLT will provide the greatest bactericidal response, due to the higher energy levels associated with the shorter wavelength photons. Furthermore, it is expected that low-irradiance LLLT will provide a greater bactericidal rate when compared to delivering the same LLLT fluence at a higher-irradiance, because of the fact that visible blue LLLT is dependent upon oxygen supplies for ROS formation. Consequently, low-irradiance LLLT will not deplete oxygen supplies as rapidly and therefore provide greater treatment efficacy.

MATERIALS AND METHODS

STUDY DESIGN:

During phase 1, cultures of *MRSA* were exposed to three separate wavelengths of visible blue light (405-nm, 422-nm and 470-nm) at an irradiance of 10.44 mW/cm^2. The cultures were exposed to LLLT for a total of 8 hours. However, aliquots from each of the exposed cultures were taken in two-hour intervals (t=2,4,6,8 hours), which was the equivalent of LLLT doses of 75 J/cm^2. These aliquots were then quantified to determine the number of colony forming units per milliliter (CFU/mL) through a standard plate counting technique using a Whitley Automated Spiral Plater (WASP). Table 1 outlines the total fluence of LLLT each culture received at each of the two-hour intervals.

Upon completion of phase 1, a prospective analysis was conducted to determine which wavelength would provide the greatest bacterial load reduction at the lowest possible fluence. After determining these conditions, phase 2 was initiated to evaluate the effects of maintaining the aforementioned optimal fluence, but further reducing the irradiance and increasing the total exposure time of LLLT to 5.22 mW/cm^2 (4 hour exposure) and 3.48 mW/cm^2 (6 hour exposure).

BACTERIAL ISOLATES:

The MRSA strain that was used in this procedure was ATCC(r) BAA-1761 (ATCC, Manassas, VA). A loopful of frozen inoculum from –80°C frozen stocks was used to quadrant streak onto the surface of a tryptic soy agar (TSA) plate. Each plate was then incubated at 37°C and 5% CO_2 for 24 hours to produce isolated and pure cultures of MRSA for this study.

BIOSAFETY PRECAUTIONS:

Throughout the course of the procedure outlined within this experimental study, adequate biosafety measures were taken to comply with the fact that MRSA is classified under the Biosafety Level 2 Risk Group. In addition to wearing laboratory coats, gloves and eye protection, all facets of this procedure were conducted using aseptic technique within a Class II laminar flow biosafety cabinet.

LIGHT EMITTING DEVICE:

The LED light sources used during Phase 1 of this study were LEDs at 405±5-nm, 422±5-nm and 470±5-nm wavelengths (Visual Communications Company, Poway, CA). The light-emitting device was constructed by assembling LEDs onto an EIC-108 3220 Tie-Points Solderless Breadboard (EIC Laboratories INC., Norwood, MA). A Labnet Power Station 300 power supply (Labnet International, Edison, NJ) was connected to provide the desired voltages to emit the necessary irradiance. 24 LEDs were assembled onto each breadboard, and were then positioned directly beneath one well on a 24-well plate, so each well would be illuminated by a single LED. To ensure that each well was receiving identical irradiances of blue light, the overall irradiance that each well was illuminated by was measured using a THORLABS Optical Power Meter (Thor Laboratories, Newton, NJ).

PHOTOINACTIVATION PROCEDURE:

MRSA was grown for 24 hours at 37°C and 5% CO_2 in tryptic soy broth (TSB). After isolating colonies of MRSA, a single colony of MRSA was suspended in TSB to grow for 24 hours. Following incubation, aliquots of the cultures were serially diluted in sterile saline to a final dilution factor of 1:10,000 (TSB suspended MRSA culture: Saline). This dilution was completed in order to quantify the bacteria within the constraints of the Whitely Automatic Spiral Plating system outlined below. The concentration of MRSA in the diluted cultures was confirmed through monitoring their optical density (OD_{600}) at 600-nm.

275.0 µL of the diluted MRSA cultures were loaded into each well of a Corning® Costar® 24-Well Flat Bottom Cell Culture Plate with a Total Well Volume of 3.4 mL (Corning Inc, Corning, NY) to receive light exposure. Throughout the course of this experiment, there were four separate trials conducted to ensure consistency, and to evaluate both the intraplate and interplate bactericidal effects of the low-irradiance LLLT. During each individual trial in phase 1, there were four experimental cohorts where MRSA cultures received either: 1) no light exposure (control); 2) 405-nm LLLT; 3) 422-nm LLLT; or 4) 470-nm LLLT. For each cohort that received

LLLT, the light was administered at a fixed irradiance of 10.44 mW/cm^2. During phase 2, there were also found experimental cohorts where *MRSA* cultures received either: 1) no light exposure (control); 2) LLLT at 10.44 mW/cm^2 over 2 hours; 3) LLLT at 5.22 mW/cm^2 over 4 hours; or 4) LLLT at 3.48 mW/cm^2 over 6 hours. During phase 2, cultures that received LLLT were treated with the optimal wavelength determined during phase 1. With the exception of the light wavelengths from the LED source used on the experimental groups, each trial was completed at room temperature in total darkness to eliminate confounding variables from external light sources. Additionally, in order to minimize the risk for contamination, the well plates remained covered for the duration of the light treatment. Throughout the course of each trial, fifty-microliter aliquots were taken from four randomly selected wells using aseptic technique, although no well was repeatedly sampled, in each well plate during increments of 2 hours of light exposure. These aliquots were then plated onto TSA using the Whitley Automated Spiral Plating system.

STANDARD PLATE COUNT PROCEDURE:

A Whitley Automated Spiral Plater (WASP) (Don Whitley Scientific Limited, West Yorkshire, UK) was used to spiral plate 50 μL of each aliquot onto the surface of a tryptic soy agar plate in an Archimedes spiral. The plates were incubated for 24 hours at 37°C in 5% CO_2. Colony counts were manually performed three times per plate, and the mean value was subsequently used to calculate the bacterial load in units of CFU/mL.

STATISTICAL ANALYSIS

Data throughout the course of this study was analyzed *post-hoc* using one-way ANOVA and Student *t*-tests. Each illumination condition was compared to their control, as well as wavelength differences between groups. P values < 0.05 were considered to be statistically significant.

RESULTS

As illustrated in Table 1 and Fig. 1, 405-nm low-irradiance LLLT provided the greatest total bacterial load reduction of *MRSA* cultures (99.06% reduction [$p < 0.05$]) in Phase 1, when compared to 422-nm (98.84%) and 470-nm (98.71%) LLLT. Furthermore, 405-nm LLLT was the only wavelength that successfully provided a 2.0-log bacterial load reduction ($\geq$ 99.0% kill rate).

While 405-nm LLLT provided the greatest cumulative bacterial load reduction among the eight-hour light exposure, Table and Fig. 1 also suggest there was a greater bacterial load reduction seen from a fluence of 75 J/cm^2 across the first two hours of 405-nm LLLT treatment (94.50% [$p < 0.0001$]) than what was seen from a fluence of 150 J/cm^2 across the first four hours of 422-nm LLLT (83.47%), as well as 225 J/cm^2 across the first six hours of 470-nm LLLT (93.95%), respectively. Based on these observations, phase 2 of this study was conducted using the optimal LLLT wavelength of 405-nm at a fluence of 75 J/cm^2.
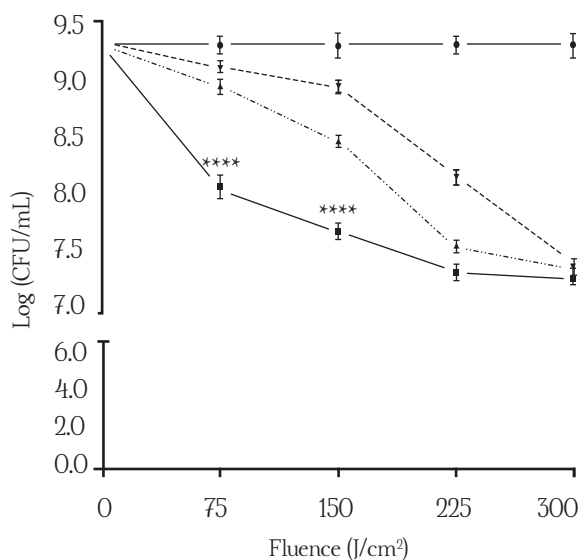
Figure 1. MRSA Colony Forming units (CFUs) Post–Low Level Light Therapy This figure is a graphical depiction of mean bacterial load reductions of MRSA following 405–nm, 422–nm and 470–nm LLLT relative to the total LLLT fluence received. Each experiment was completed at a fixed irradiance of 10.44 mW/cm^2. MRSA samples were collected in increments of 75 J/cm^2 (every 2 hours of the 8–hour treatment) of LLLT. Error bars represent standard error of collected samples. Asterisks (****) signify p–value < 0.0001 following ANOVA to analyze significant differences between experimental wavelengths.

Fluence (J/cm^2)	405–nm		422–nm		470–nm	
	log (± SEM)	Percent Change	log (± SEM)	Percent Change	log (± SEM)	Percent Change
75	8.06 (± 0.03) [p < 0.0001]	94.50%	8.93 (± 0.02)	54.32%	9.10 (± 0.02)	36.87%
150	7.67 (± 0.02) [p < 0.0001]	97.53%	8.46 (± 0.02)	83.47%	8.93 (± 0.03)	53.42%
225	7.31 (± 0.02) [p <0.021]	98.93%	7.54 (± 0.02)	96.92%	8.15 (± 0.02)	93.95%
300	7.26 (± 0.02) [p <0.05]	99.06%	7.36 (± 0.02)	98.84%	7.39 (± 0.02)	98.71%

Table 1. Bacterial Load Reduction of MRSA Samples Following 10.44 mW/cm^2 LLLT: This table indicates the mean bacterial load reductions log(CFU/mL)(± SEM) of MRSA following low–irradiance LLLT at wavelengths of 405–nm, 422–nm and 470–nm. All samples were exposed to light at a constant irradiance of 10.44 mW/cm^2, but received LLLT at varying exposure times and total fluences (J/cm^2). A total of 16 replicates (4 from each experimental trial) of each data point were collected to determine mean bacterial load reductions. P–values signify level of significance differences among the experimental wavelengths using ANOVA. P–values < 0.05 are considered significant.

Irradiance (mW/cm^2)	LLLT Exposure Period	Mean Log (± Standard Error)	Mean Bacterial Load Reduction	P-value
10.44	2 hours	8.06 (± 0.03)	94.50%	–
5.22	4 hours	7.91 (± 0.04)	95.71%	< 0.023
3.48	6 hours	6.85 (± 0.03)	99.63%	< 0.0001

Table 2: Bacterial Load Reduction of MRSA Following Irradiance-Manipulated 405-nm LLLT: This table indicates the mean bacterial load reductions (CFU/mL) of MRSA following 75 J/cm^2 of 405-nm low-irradiance LLLT. While all samples received a constant fluence of 75 J/cm^2, the irradiance and exposure times were manipulated from 10.44 mW/cm^2 to 5.22 mW/cm^2 and 3.48 mW/cm^2. A total of 16 replicates (4 from each experimental trial) of each data point were collected to determine mean bacterial load reductions. P-values signify level of significance differences among the experimental irradiances using ANOVA. P-values < 0.05 are considered significant.

Upon determining the optimal fluence and wavelength of LLLT in Phase 1, Phase 2 was conducted to analyze 405-nm LLLT when delivering the same total fluence of 75 J/cm^2, but through irradiances of 5.22 mW/cm^2 and 3.48 mW/cm^2. By decreasing the irradiances, the duration of each LLLT cycle to receive 75 J/cm^2 was increased from the initial two-hour treatment at 10.44 mW/cm^2 to 4 hours at 5.22 mW/cm^2 and 6 hours at 3.48 mW/cm^2.

Table 2 indicates that there was a correlation associated with a greater bacterial load reduction when an identical fluence of 75 J/cm^2 405-nm LLLT was administered at a lower irradiance and subsequently an increased exposure time. An increased bacterial load reduction was observed when the irradiance was decreased to 5.22 mW/cm^2 (95.71% reduction [p < 0.023]) and 3.48 mW/cm^2 (99.63% reduction [p < 0.0001]).

DISCUSSION

One significant limitation of conventional LLLT is that the high-intensity light sources that are commonly used to administer therapy are relatively expensive, and require a specialized medical facility and staff to operate and maintain. Consequently, these centers require frequent travel for patients that are looking to receive LLLT, and may not be readily accessible. In order to facilitate introducing LLLT into mainstream medicine, recent studies have analyzed the use of low-power Light Emitting Diodes (LEDs) as an alternative illumination source to the high-powered lasers that are currently used (1, 20, 27).

This in vitro study validates the proof of concept of the bactericidal effects of low-irradiance LLLT using visible blue low-powered LEDs on cultures of MRSA. Photoinactivation of MRSA was achieved through LLLT at wavelengths of 405-nm, 422-nm and 470-nm, as seen in precedent

literature (1,10,19–20,23,27).). However, it was observed that the optimal wavelength for visible blue LLLT was 405–nm (p < 0.0001).. The results of this study are consistent with the findings of Bumah *et al*., which illustrated that 405–nm photoinactivation resulted in a more efficient colony count reduction as opposed to other wavelengths within the visible blue spectrum (6).

While multiple studies have confirmed the efficacy of LLLT at one discrete wavelength or fluence, this study intended to evaluate the photoinactivation rates of multiple wavelengths to determine the optimal wavelength and fluence of LLLT within the visible blue spectrum. As seen in Table 1 and Fig. 1, 405–nm photoinactivation effectively illustrated a more immediate bactericidal effect compared to its 422–nm and 470–nm counterparts. Despite achieving similar bacterial load reductions at each wavelength following the entire 300 J/cm^2 light treatment, 405–nm LLLT resulted in a 94.50% load reduction following its initial 75 J/cm^2 (2 hours) of irradiation. Based on the parameters of this phase 1 study, 75 J/cm^2 of 405–nm blue light was determined to be the optimal blue light dosage, and was therefore held constant throughout phase 2.

Following phase 1, the irradiance (mW/cm^2) at which the light was delivered to the *MRSA* cultures was manipulated during phase 2 to determine if a lower, more continuous light exposure could result in a maximum formation of reactive oxygen species (ROS) through a specific fluence. As illustrated in Table 2, low–irradiance LLLT provided a statistically significant improvement [*p* < 0.0001] in reducing the bacterial load of *MRSA*, and should be considered as a viable, therapeutic alternative to high intensity LLLT, which conversely deliversthe total fluence over short durations, ranging from 60 seconds to 10 minutes.

Based on this principle, LLLT illumination using a high irradiance light source could potentially deplete oxygen supplies more rapidly than low–irradiance LLLT, and therefore compromise the efficacy of the intervention (4, 38). Future studies using this low–irradiance strategy will add merit to the growing body of evidence of the efficacy and safety of LLLT.

One of the limitations of this study was the inability of the LED apparatus to administer a uniform irradiance of less than 3.48 mW/cm^2 to each of the 24–wells due to a constraint of the power supply used in this experimental setup. Based upon the parameters of this study, subsequent studies should be completed to evaluate the bactericidal effects of LLLT using irradiances lower than the 3.48 mW/cm^2 to determine if there is a minimum threshold to low–irradiance that would consequently compromise efficacy of treatment. Another limitation of the current study was that the bactericidal effects of low–irradiance LLLT were only evaluated on one strain of *MRSA*. Studies completed by Grinholc *et al*. (13) determined after analyzing over 40 distinct strains of *MRSA* that the bactericidal effect of LLLT was strain–dependent, with bacterial load reduction counts ranging from 0% – >99.9% (3 log–reduction) (13). While this study did analyze the effects of LLLT using a light source in the visible red (624–nm) spectrum, subsequent analyses of the bactericidal effects of 405–nm low–irradiance LLLT should be evaluated among various Gram–positive and Gram–negative organisms, and their respective strains.

In addition to subsequent studies strictly analyzing the efficacy of 405–nm low–irradiance LLLT for its bactericidal and wound healing capabilities, there is a growing body of evidence that LLLT using multiple wavelengths has provided

synergistic effects that benefit the overall therapeutic outcomes when compared to using solely monochromatic LLLT (12, 14, 18, 21–22). Studies from Goldberg *et al.* (12) and Lee *et al.* (18) observed the efficacy of visible blue (415±5 nm) and red (633±6 nm) combination light for the treatment of acne vulgaris in a 27 patient study, and determined that phototherapy using multiple wavelengths was an effective strategy to reduce bacterial load as well as accelerate skin healing (12, 18). Translating the efficacy of combination wavelength treatments to other areas of medicine could possibly reduce the potential of oral tissue infection following oral surgery while simultaneously promoting faster healing of gingival tissue or additional soft tissues within the oral cavity. Furthermore, combination wavelength treatments show promise in dermatologic, surgical, and military environments where infection management, tissue rescue, and tissue healing are directly connected to survival or other favorable outcomes.

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