Research Article

Inhibition of *Mycobacterium smegmatis* using near-IR and blue light

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ABSTRACT

Background: Objective of current study is to determine whether near-IR, alone or combined with blue light and delivered at a low rate, could lower the dose needed to effectively inhibit *Mycobacterium smegmatis in vitro*. We have studied the effect of blue light on *M. smegmatis* and found that a bactericidal outcome can be obtained with high doses of blue light.
Methods: The organism was treated *in vitro* with 464, 850 and combined 464 & 850nm light emitted from a supraluminous diode (SLD) array. Doses of 30, 45 and 60 J/cm^2 were used. Colony counts were performed and compared to untreated controls using Student t tests, a two-way Repeated Measures ANOVA and a one-way ANOVA with Tukey post hoc analysis.
Results: Statistically significant inhibition was observed for each individual wavelength and dose combination ($p < 0.05$). Two-way ANOVA demonstrated an interaction effect between wavelength and dose ($F_{1, 9} = 358.585$; p - 0.000). Post hoc analysis using one-way ANOVA ($F_{2, 27} = 11.211$; $p = 0.00$) and Tukey's HSD identified 850nm at 45 J/cm^2 to be the most effective wavelength / dose combination.
Conclusions: 850nm irradiation delivered at 45 J/cm^2 is a wavelength /dose combination that can be expected to produce a significant inhibition of *M. smegmatis in vitro*.

Keywords: Near-IR, *Mycobacterium*, Bactericidal effect

INTRODUCTION

For several decades antibiotics have been the treatment of choice for fighting infections.[1] As effective as antibiotics have been, indiscriminant use in humans and widespread application in agriculture have led to the development of antimicrobial resistance.[2] The era of antibiotic use may be approaching its end.[3] The New Delhi Metallo-Beta-Lactamase-1 (NDM-1) gene, an enzyme that renders some bacteria resistant to almost all antibiotics, has been found in the United States.[4] The appearance of the NDM-1 gene has supported the concern that bacteria associated with infectious diseases will become increasingly unresponsive to antibiotic treatment.[3] This concern has led to increased interest in alternative therapies to antibiotic use and light therapy may represent one of those alternatives. Enwemeka recently editorialized light as an alternative modality in the current paradigm shift away from pharmaceutical treatment.[5]

The application of light energies, as an alternative to antibiotics, is promising because light can be easily applied, the dose and delivery are controllable and predictable, and its potential effectiveness is supported by a growing amount of evidence in the literature. Blue light (400 – 495 nm) inhibits *Pseudomonas aeruginosa*,[6-8] *Staphylococcus aureus*[7-9] and *Mycobacterium smegmatis*.[10] The effectiveness of blue light as a bactericidal agent appears to be enhanced when combined with IR wavelengths.[7-9] Combined red (624 nm) and IR (850 nm) wavelengths are effective against the growth of Candida albicans.[11,12] The source of the antimicrobial effect associated with

visible and IR energies is a photobiomodulation where mitochondrial chromophores in the treated organism absorb light photons and produce singlet oxygen or free radicals that are lethal to the microbe.[13]

Two other forms of light energy commonly employed for their antimicrobial effect include photodynamic therapy (PDT) and ultraviolet C (UVC) light. PDT requires the addition of exogenous photosensitizers. UVC light is known to inhibit bacterial growth, but has produced detrimental effects such as burns, premature aging, suppression of the immune system, and even skin cancers on mammalian cells and host tissue.[14] Visible and IR light, to our knowledge, have not been shown to include risks to the host tissue when delivered at commonly effective dose levels.[6,15]

Infrared wavelengths, when combined with blue light, have been shown to enhance bactericidal outcomes against Staphylococcus aureus[7-9] and *Klebseilla pneumonia*.[16] Similarly, the combination of IR (850 nm) and red (624 nm) wavelengths is effective against the growth of Candida albicans.[11,12] While these experiments were performed *in vitro*, there is evidence to support the connection between *in vitro* experiments related to bacterial inhibition and potentially effective methods for *in vivo* application.[6] The source of the antimicrobial effect associated with infrared wavelengths, as in blue light, is the absorption of light photons and production of singlet oxygen or free radicals that are lethal to the microbe.[13] *M. smegmatis*, like other Mycobacteria, has a very unique cell wall that is lipid-rich and hydrophobic . The high lipid content, which includes mycolic acids, supports resistance to the harmful effects of acids, alkalis, desiccation, and human immune defenses. It is possible that the IR wavelength alters these cell wall properties, allowing a greater potential for the light energy to be absorbed.[17]

As mentioned, studies have shown that blue light can effectively inhibit the growth of *M. smegmatis* in vitro.[10,18] The dose level needed, however, to effectively inhibit *M. smegmatis* has been significantly greater than that required with other bacteria. An approximately 100 J/cm² dose is needed to inactivate this organism.[10,18] When a dose between 144 J/cm² and 288 J/cm² was administered, 405 nm blue light was shown to inactivate M. terrae by 4-5 log10 (CFU/mL)[18] The bacterial inhibition in these studies was very high (in some cases 100%), but the dose required might exceed safe levels of exposure for the host.

The purpose of this experiment was to determine whether a combination of blue and IR wavelengths could not only be an effective inhibitor of *M. smegmatis*, but also yield this effectiveness at lower (more clinically acceptable) doses. The experiment also sought to determine whether rate of energy delivery might impact bactericidal outcomes.

METHODS

The genus-species of the organism tested was M. smegmatis. This organism grows well in ambient air. The organism was obtained from a 20-h-old culture. A suspension equivalent to a 0.5 McFarland Standard was prepared. Use of a 20-h-old culture is standard microbiological practice and serves to minimize the lag time for new growth. The suspension was further diluted 1/1000 using 100 microliter automatic pipettes for purposes of accuracy and reproducibility. All dilutions were made immediately before the treatment with light.

The dilutions were placed in sterile petri dishes (60 X 15 mm) and irradiated with 464nm, 850nm and combined 464 & 850nm light energy at 30, 45 and 60 J/cm². The light energy was delivered at a rate of 16.7 (464nm, 850nm) to 19.88 (464 & 850nm combination) mW/cm² until the various doses were achieved.

As each light energy dose was achieved, 10 microliter aliquots of the 1/1000 dilution of *M. smegmatis* were extracted from the treated dilution and inoculated onto Middlebrook 7H10 agar plates (60 X 15 mm). Middlebrook 7H10 is a chemically defined medium formulated to supply the growth factors required for *Mycobacterium* species. Mature colonies are obtained sooner on Middlebrook 7H10 agar than on egg-based media such as Lowenstein-Jensen slants. The microorganism was applied to the surface of the agar plates in a star-streak pattern. After 72 hours of incubation, colony counts were performed to compare treated and control plates.

The treated and control plates were incubated at 35°C under aerobic conditions. Compared to other mycobacteria such as *M. tuberculosis*, which may require 2-3 weeks for mature colonies to develop, *M. smegmatis* is considered to be a rapid-grower (3-5 days for mature colonies). Because *M. smegmatis* is non-pathogenic and grows rapidly it is the model species for use in mycobacterial research. It can be readily distinguished from contaminating microbes due to the characteristic macroscopic morphology on the Middlebrook 7H10 agar plates. *M. smegmatis* forms rough, raised, non-pigmented, friable colonies, which some have likened to "bread crumbs". Most contaminating organisms would form smooth, raised, circular colonies with a butyrous consistency.

We chose to illuminate the cultures using a pair of SLD light pads that emitted a band of light focused around the primary wavelengths of 464nm and 850nm. These wavelengths could be delivered individually or in combination. The pads consisted of a 353 cm² illuminating surface area comprised of 176 SLDs with a maximum power output of 5160 mW. Dose was calculated in J/cm². Since output for the light pads was held constant, adjustment in time of irradiation provided the dose (30, 45 and 60 J/cm²). After 72 hours of incubation, colony forming units (CFUs) were counted and compared to controls.

RESULTS

Data were descriptively analysed by producing a kill rate value. Kill rate was determined by subtracting the mean CFUs for treated samples from the mean CFUs for the controls. The result was divided by the control CFU mean and multiplied by 100 to produce a percentage value ([Control – Treated]/Control X 100).

Next, a paired student-t test was used to determine whether the kill rate obtained in any condition (wavelength and dose) was statistically significant. Table 1 displays the result of this initial analysis. Each of the wavelength / dose combinations produced a significant ($p < 0.05$) kill rate.

A two-way repeated measures ANOVA was performed to determine the main and interactive effects of wavelength and dose in terms of kill rate observed. Since significant effects were observed ($F_{1,9} = 358.585$; $p = 0.000$), a one-way ANOVA ($F_{2, 27} = 11.211$; $p = 0.00$) and post hoc Tukey's Honest Significant Difference were employed to identify the homogenous subsets. The post hoc analysis identified 850nm wavelength at 45 J/cm^2 to be significantly different from the other wavelength/dose combinations. Figure 1 graphically demonstrates these outcomes. SPSS 20 was the software package employed for the data analysis.

Table 1: Kill rates by dose and wavelength for *M. smegmatis*.

Wavelength (nm)	Dose (J/cm^2)	N	Control (Mean CFU)	Treated (Mean CFU)	Kill Rate (%)	*p* Value
464	30	10	48.3	30.90	36.02	0.000
464	45	10	48.3	30.70	36.44	0.000
464	60	10	48.3	31.2	35.40	0.007
850	30	10	42.8	19.1	55.37	0.000
850	45	10	42.8	12.5	70.79	0.000
850	60	10	42.8	17.3	59.58	0.000
464 & 850	30	10	48.3	25.6	46.99	0.000
464 & 850	45	10	48.3	29.3	39.34	0.000
464 & 850	60	10	48.3	27.2	43.69	0.000

nm = Nanometers
J/cm^2 = Joules per square centimeter
CFU = Colony Forming Units
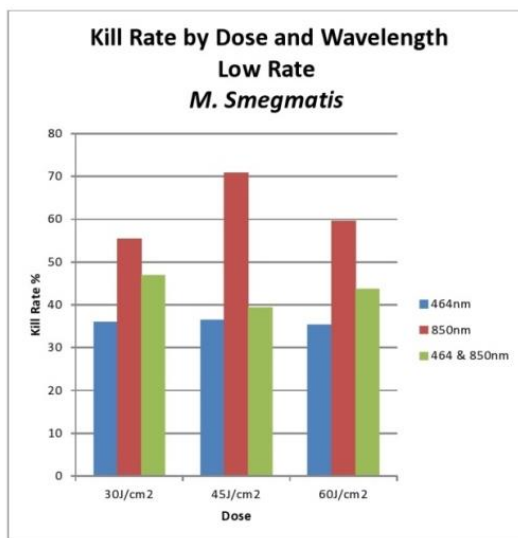
Figure 1: Kill rate by dose and wavelengh low rate *M. smegmatis*.

DISCUSSION

The cell wall of mycobacteria is truly unique. This cell wall makes the organism extremely hardy and able to thrive in environments hostile to other bacteria. Antibiotics that effectively kill other bacteria have little or no effect on mycobacteria. An entirely different class of antimicrobial agents is needed to treat mycobacterial infections. These medications must be taken for a period of 6 months to one year to be effective. Many patients take the drugs for a short while and then stop taking the medication, allowing resistant strains to develop. This departure from recommended drug use is one factor contributing to the presence of multiple-drug resistant strains of tuberculosis (MDR-TB).[19]

Our earlier work with *M. smegmatis* demonstrated the effectiveness of blue light as an inhibitor of this organism.[10] The dose required to produce an effective inhibition was quite high (at least 120 J/cm^2). Our

concern was that doses high enough to produce inhibition *in vitro* (using blue light) might not be safe in terms of the host tissues if delivered in an *in vivo* situation. Adding a near-IR wavelength and lowering the rate of delivery (<20 mW/cm^2 in this experiment versus >100 mW/cm^2 in our earlier work) appear to be techniques that can result in a lower effective dose.

In some of our earliest work we found that the addition of IR energy could enhance bacterial inhibition.[8] We chose to include IR in these earlier experiments because of the work done by Karu.[17] Recently, Lee et al. have also demonstrated the photobiomodulation derived inhibition potential associated with IR.[13] For *M. smegmatis*, the near-IR wavelength was superior, in terms of inhibition, to either blue (464nm) light alone or to a combination of blue and near-IR (464 & 850nm) light. This experiment was not intended to particularly identify the mechanism(s) that produce inhibition of *M. smegmatis*. We sought to determine whether it was possible to lower the dose required to achieve inhibition. Incorporating a near-IR wavelength did produce an inhibition at a dose lower than observed in our previous experiments. We would refer to Karu to offer possible explanation.[17] Visible and near-IR radiation may produce changes in the respiratory components of the cell, generate singlet oxygen, generate superoxide ions and even heat chromophores that absorb the near-IR energy. Any, all or none of these factors may be the reason we saw improvements in outcome when IR was added. Others may be better suited to identifying the mechanisms involved.

The particular physiology of each organism allows it to exploit a unique niche in the environment (be it soil, water or inanimate objects) or the body of the host. For years microbiologists have been aware that physiologic differences play a role in the ability of microorganisms to evade host defences and cause disease. Structures such as capsules and fimbriae or components of the cell wall that enhance the ability of an organism to initiate an infection (its virulence factors) must be specifically addressed in the design of antibiotic drugs.[20] It is possible that those same specific virulence factors may speak to why a given wavelength, dose and/or rate of energy delivery is specific to an organism in terms of inhibition.

CONCLUSIONS

Based on the data collected in this experiment, we are able to draw the following conclusions.

1. It is possible to obtain an effective inhibition of *M. smegmatis* using light energy at lower dose levels than previously reported.[10, 18]
2. While blue light has been shown to effectively inhibit *M. smegmatis*, near IR wavelengths may also produce an effective inhibition.

3. 850nm irradiation delivered at 45 J/cm^2 is a wavelength /dose combination that can be expected to produce a significant inhibition of *M. smegmatis in vitro*.

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