Abstract: Objective. To determine whether manipulation of dose, wavelength, and rate of energy delivery could delay the onset of previously demonstrated Staphylococcus aureus resistance to blue light. Methods. The organism was treated in vitro with 405 nm, 464 nm, and combined 464 nm and with 850 nm light emitted from a supraluminous diode (SLD) array. Doses of 9 J/cm² and 30 J/cm² were used. Rates of energy delivery were also varied from 10 mW/cm² to 125 mW/cm². Seven stages were employed to test for resistance formation. Colony counts were performed and compared to untreated controls using Student t tests and one-way ANOVA with Tukey post hoc analysis. Results. A best dose, wavelength, and rate of delivery combination was determined at each stage and it did produce a significant kill rate ($P \leq 0.05$) at each stage. Analysis of variance demonstrated that no loss of effectiveness (formation of resistance) occurred over the 7 stages. Conclusions. Appropriate combinations of dose, wavelength, and rate of energy delivery can delay resistance formation to light as a bactericidal agent for S. aureus.

Key words: blue light, Staphylococcus aureus, resistance formation

Antimicrobial resistance has emerged as one of the major challenges facing clinicians tasked with treating skin breakdown.\textsuperscript{1,2} Pharmaceutical agents used to treat infection have made a significant contribution to human health,\textsuperscript{3} but this demonstrated resistance capability threatens the long-term facility of antibiotics. As Bush et al\textsuperscript{4} have pointed out, finding alternative antimicrobial agents/techniques must be a high-priority focus of current research.

Ultraviolet light, electrical currents, and visual light are among the list of nonpharmaceutical agents that have been used to successfully inhibit the growth of infectious agents. While ultraviolet light has been demonstrated to be an effective bactericidal agent,\textsuperscript{5,6} it includes the potential
of detrimental effects to human tissue. High voltage pulsed electrical stimulation is effective in inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, but the treatment time required for clinical effectiveness is quite long. Visible light has been shown to have bactericidal effects on microbial organisms, however the results tend to depend on the organism treated and wavelength employed.

Photodynamic therapy (PDT) is a form of light therapy that requires the addition of a photosensitizing dye. After the dye has been absorbed, the microbe is irradiated with visible and/or infrared light. While PDT can produce significant bactericidal inhibition, the technique requires the microbial cells to absorb the dye. The combination of photosensitizer and light can be a challenge due to competition for the photosensitizer between microbial cells and host tissue.

Advantages of the light-based antimicrobial treatment include equal killing effectiveness regardless of the antibiotic resistance. Guffey and Wilborn demonstrated that *Staphylococcus aureus* and *P. aeruginosa* were effectively inhibited by blue light (405 nm) and green light (510 nm). The current study focused on developing methods to retard the resistance capability of *S. aureus* demonstrated.

Methods and Materials

*S. aureus* ATCC 25923 was the organism used in this study. It was harvested from a 20-hour-old culture and added to sterile deionized water to form a suspension equivalent to a 0.5 McFarland Standard. Use of a 20-hour-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 diluted solution was then exposed, in each stage, to the various doses and wavelengths of light. In each case (probe and pads) the light source was brought as close to the suspension as possible without contacting the liquid (< 5 mm). The distances were consistent for each exposure.

Using a 10-microliter automatic pipette, an aliquot of the 1/1000 dilution of *S. aureus* was inoculated onto Mannitol salt agar (MSA) in 60 mm X 15 mm sterile, polystyrene petri dishes. Mannitol salt agar was chosen as a growth medium because it is a selective and differential medium. The conditions and environment in which the experiment was conducted were not sterile. The authors chose to use the selective and differential medium to avoid contaminants from growing on the plates and confusing the colony counts 24 hours later. The differential medium made the colonies easier to count. Mannitol salt agar is selective for organisms able to grow in 7.5% (w/v) NaCl (primarily *Staphylococcus* species). The medium is also used to differentiate mannitol fermenters (primarily *S. aureus*) from non-mannitol fermenters (coagulase negative staphylococci).

It is already documented that blue light can inhibit the growth of *S. aureus*. Recently, the ability of *S. aureus* to develop resistance to 405 nm light when that light energy is administered in more than 5 successive stages, using only the single wavelength, a fixed dose, and a rather high rate of delivery, was demonstrated. The present experiment sought to identify a protocol that might slow this organism’s ability to form a resistance to the light energy. To explore this possibility, the
S. aureus, prepared as described above, was exposed to 405 nm light at doses of 9 J/cm² and 30 J/cm². This represented stage 1 of the experiment. The S. aureus was then inoculated onto the mannanol plates and incubated for 20-24 hours at 37°C. Colony forming units (CFU) were then counted. Organisms that were able to survive the best kill rate from the stage 1 treatment with light at a wavelength of 405 nm were subcultured to blood agar plates so they could be subjected to a subsequent exposure of light. The surviving organisms were subcultured to the blood agar so that a smooth suspension could be obtained for the next stage of the experiment. Taking organisms directly from the MSA to prepare the suspension resulted in difficulty obtaining a sample that would be homogeneous in solution.

Organisms were removed from the blood agar plate and a suspension equivalent to a 0.5 MacFarland standard was prepared. The bacterial suspension was further diluted 1/1000 using 100 microliter automatic pipettes to maintain accuracy and reproducibility. This solution containing S. aureus was then subjected to blue and infrared light. In this case, stage 2, the microorganism was subjected to the light at a combined 464 nm and 850 nm delivered at 9 J/cm² and 30 J/cm².

The experiment proceeded using these methods for a total of 7 stages. A summary of the doses, wavelengths, and exposure times at each stage are provided in Table 1. After each exposure and incubation at 37°C in ambient air, a colony count was performed to determine the kill rate. If the method of light exposure was effective in terms of slowing resistance formation, a consistent kill rate should have resulted across the 7 stages.

Light exposures were achieved using an electrotherapy tri-wave light ultrasound device (Dynatron 705 Plus Solaris Dynatronics Corp, Salt Lake City, UT). This device is designed to accommodate both light probes (higher rate of delivery) and light pads (lower rate of delivery). For this experiment, the authors chose to illuminate the cultures using a supraluminous diode (SLD) light probe that emitted a focused band around the primary wavelength of 405 nm (stage 1) and a pair of SLD light pads that emitted a band of light focused around the primary wavelengths of 464 nm and 850 nm. The probe consisted of a 5 cm² illuminating surface area comprised of 34 SLDs with a maximum power output of 1000 mW. The pads consisted of a 353 cm² illuminating surface area comprised of 176 SLDs with a maximum power output of 1000 mW. Dose was calculated in J/cm². Since output for the probe was held constant, adjustment in time of irradiation provided the dose (9 J/cm² or 30 J/cm²). The electrotherapy tri-wave light ultrasound device automatically calculates time of irradiation when desired dosage is selected. Figures 1 and 2 show a visual display of the light energy delivery.

### Results

Significant ($P < 0.05$) kill rates were obtained at each stage of the experiment. This result was confirmed by performing dependent $t$ tests at each stage. Secondly, resistance development was not observed. This result was confirmed by an ANOVA ($F_6, 28 = 9.381; P < 0.0001$) whose Tukey’s post hoc analysis identified only stage

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**Table 1. Light exposure details.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Wavelength</th>
<th>Dose</th>
<th>Delivery rate</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>405 nm</td>
<td>9 J/cm²</td>
<td>125 mW/cm²</td>
<td>1 min, 48 sec</td>
</tr>
<tr>
<td>2</td>
<td>464 nm &amp; 850 nm</td>
<td>9 J/cm²</td>
<td>10 mW/cm²</td>
<td>15 min, 4 sec</td>
</tr>
<tr>
<td>3</td>
<td>464 nm</td>
<td>9 J/cm²</td>
<td>20 mW/cm²</td>
<td>7 min, 32 sec</td>
</tr>
<tr>
<td>4</td>
<td>464 nm &amp; 850 nm</td>
<td>9 J/cm²</td>
<td>20 mW/cm²</td>
<td>9 min, 30 min</td>
</tr>
<tr>
<td>5</td>
<td>464 nm</td>
<td>9 J/cm²</td>
<td>20 mW/cm²</td>
<td>7 min, 32 sec</td>
</tr>
<tr>
<td>6</td>
<td>464 nm &amp; 850 nm</td>
<td>9 J/cm²</td>
<td>20 mW/cm²</td>
<td>9 min, 30 min</td>
</tr>
<tr>
<td>7</td>
<td>464 nm</td>
<td>9 J/cm²</td>
<td>20 mW/cm²</td>
<td>9 min, 30 min</td>
</tr>
</tbody>
</table>

| nm = nanometers; J/cm² = Joules per square centimeter; min = minutes; sec = seconds

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**Keypoints**

- The experiment sought to identify a protocol that might slow the ability of S. aureus ATCC 25923 to form a resistance to the light energy.
- The authors employed 2 known effective doses at each of 7 stages to determine whether varying the dose from stage to stage would delay the formation of resistance.
5 (significantly greater kill rate) as being different from the other stages. The kill rate, rather than declining over the stages, stayed at or above the initial rate obtained in stage 1. The average kill rate for the 7 stages was 51.22%. Table 2 provides specifics related to these data with the most effective combination of wavelength(s), dose, and rate of delivery listed.

**Discussion**

Antimicrobial resistance formation is a challenge that threatens the long-term viability of any treatment regimen. It has previously been demonstrated that *S. aureus* has the ability to become resistant to blue light if that light energy is not manipulated. In the current study, the authors employed 2 known effective doses at each stage to determine whether varying the dose from stage to stage would delay the formation of resistance. Visible and infrared wavelengths were also intermittently combined and the rate of delivery of the light energy was varied.

Varying the rate of delivery may be an important consideration, not only for delaying the onset of resistance, but also for increasing effectiveness of treatment. The authors' thinking on this point was that lowering the rate of delivery might increase the potential for more of the dose to be absorbed. Assuming that only the absorbed energy could produce an effect (kill rate), lowering the rate of delivery could possibly improve the overall energy absorption. A loss of effectiveness over the 7 stages was not seen. Whether rate of delivery was the key factor remains unclear, but the authors plan to continue studying this possibility.

The 3 different rates of delivery (125, 20, and 10 mW/cm²) were chosen because they represent a range of delivery rates and were easily achieved with the instrumentation used in the study.

<table>
<thead>
<tr>
<th>Stage</th>
<th>N</th>
<th>Control (SD)*</th>
<th>Treated (SD)*</th>
<th>Kill Rate %</th>
<th>Device</th>
<th>Dose*</th>
<th>Wavelength**</th>
<th>Rate***</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>35.6 (10.9)</td>
<td>20.0 (7.2)</td>
<td>43.82</td>
<td>Probe</td>
<td>30</td>
<td>405</td>
<td>125</td>
<td>0.007</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>52.2 (12.8)</td>
<td>40.0 (5.8)</td>
<td>37.50</td>
<td>Pads</td>
<td>30</td>
<td>464 &amp; 850</td>
<td>10</td>
<td>0.004</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>42.8 (11.5)</td>
<td>26.2 (5.5)</td>
<td>38.78</td>
<td>Pads</td>
<td>30</td>
<td>464</td>
<td>20</td>
<td>0.009</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>35.0 (9.3)</td>
<td>20.6 (6.02)</td>
<td>41.14</td>
<td>Pads</td>
<td>9</td>
<td>464 &amp; 850</td>
<td>20</td>
<td>0.004</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>34.0 (8.1)</td>
<td>5.00 (5.2)</td>
<td>85.29</td>
<td>Pads</td>
<td>30</td>
<td>464</td>
<td>20</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>31.8 (8.3)</td>
<td>10.4 (5.7)</td>
<td>67.30</td>
<td>Pads</td>
<td>30</td>
<td>464 &amp; 850</td>
<td>20</td>
<td>0.000</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>20.6 (5.5)</td>
<td>11.4 (8.9)</td>
<td>44.70</td>
<td>Pads</td>
<td>30</td>
<td>464</td>
<td>20</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Mean of Colony Forming Units (CFU); *Joules per centimeter squared (J/cm²); **Nanometers (nm); ***Rate of Energy Delivery – milliwatts per centimeter squared (mW/cm²).
Infrared light energy (850 nm) combined with blue light in alternate stages was included. A previous study by the author using blue light included infrared wavelengths and the authors elected to return to this combination method. While the unpublished trials have shown that infrared alone is not particularly effective as a bactericidal treatment, when combined with blue light, it can enhance and sustain outcomes.

Whether these in vitro outcomes can be generalized to the in vivo environment is an important question. The argument can certainly be made that the natural biofilm that is part of the in vivo wound environment could alter/effect the way light impacts microbiota. However, Dia et al has shown that techniques similar to those used in this study are effective at decontaminating mice burn wounds infected with P. aeruginosa. It appears that results from in vitro experiments may be similar to in vivo examples.

Additional research is needed to arrive upon the most effective treatment protocol to both produce a bactericidal outcome and delay the onset of resistance formation. The authors’ current ongoing research is suggesting that each type of microorganism responds uniquely to light energy.

Conclusions
The data collected in this research have led the authors to draw the following conclusions: 1) It is possible to delay the onset of resistance formation when using light energy as a bactericidal agent for S. aureus; 2) varying the dose over successive stages between 30 J/cm² and 9 J/cm² is a possible technique for delaying resistance formation; 3) including infrared wavelengths in combination with blue wavelengths may assist in delaying resistance formation; and 4) rate of energy delivery should be further studied. Lower rates of delivery may be more effective when attempting to decontaminate tissues colonized by S. aureus. The authors have already begun to examine the interactions between dose, rate of delivery, and wavelength combinations. Further research into how these variables impact treatment outcomes is indicated.

References


