Treatment of infected wounds by using antimicrobial blue light phototherapy

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Abstract
We aimed to investigate the effects of blue light LED in treating infected wounds. Thirty male mice were divided into G1 (control) and G2 (treated). Two circular excisional skin wounds on the animal's back were made. Infection was created in right wounds 24 hrs. PW by inoculating 0.2 mL Pseudomonas aeruginosa bacterial suspension at dose 2*10^8 CFU/mL. Left wounds did not inoculate and were left untreated under the same animal control. Infected wounds in G1 were not treated; in G2, they were treated with blue light LED 420 nm 30 J/cm^2, single dose/day, for seven successive days. Healing was assessed by measuring the size of the wound, wound bacterial count, and histopathological biopsies obtained at 7- and 14-days PI. The size of the infected wounds in G1 become significantly larger 129.35 and 174.66% at 7- and 14-days PI, respectively. The infection in treated wounds of G2 was eradicated, and the size of wounds was significantly reduced 46.38, and 42.66% at 7- and 14-days PI, respectively. Numbers of bacterial colonies in treated wounds of G2, seventh day PI were expressively reduced at the second dilution. Histopathologically, infected wounds of G1 at 7- and 14-days show suppurative exudate, dead liquefied tissue, and dead and live neutrophils. Treated wounds of G2 at seven days PI, display epithelialization of the epidermis and immature granulation tissue. At 14 days PI, shows a well-regenerated epidermis and mature collagen fibers in the dermis. In conclusion blue LED treatment effectively eradicates P. aeruginosa infection and improves healing.

Keywords: Blue light, Phototherapy, Pseudomonas aeruginosa, Antibiotics resistance, Wound

Introduction
Skin is the largest organ in the body, accounting for 15% of total adult body weight. It performs vital functions such as protection against external, physical, chemical, and biological attackers via an integumentary system composed of three layers: the epidermis, dermis, and subcutaneous tissue (1). Wound healing is an essential but complicated process in humans and animals, with multiple phases governed by sequential yet overlapping phases such as hemostasis/inflammation, proliferation, and remodeling (2). Pseudomonas aeruginosa is a Gram-negative bacillus widely distributed in the environment. It has been isolated from a variety of animal species (3), meat (4,5), and fish (6). It is commonly associated with opportunistic infection but can also be found in otherwise healthy patients. P. aeruginosa infections can range from minor skin infections to life-threatening systemic disease (7). P. aeruginosa is the main bacterial type that contributes to the contamination of wounds and burns and causes wound infection (8,9). Pseudomonas aeruginosa is highly resistance to antibiotics. Pseudomonas aeruginosa strains are well recognized for using their high levels of intrinsic and acquired resistance mechanisms to fend off the majority of antibiotics (10-12). P. aeruginosa contains various virulence mechanisms that boost its capacity to inflict severe infections, including secretion of toxins, quorum sensing, and biofilm formation (13). In order to overcome P. aeruginosa, various therapeutic approaches must be developed and discovered (14). The use of blue light is one of these procedures. A certain anti-
Pseudomonas aeruginosa biofilm development has been successfully inhibited by light at 410 nm (15). Blue light (BL) 405-470 nm is naturally antimicrobial without the use of exogenous photosensitzers (PS), and it is less harmful to mammalian cells than UV exposure (16,17). Blue light is used as a part of antimicrobial phototherapy. Blue light displays a broad-spectrum antimicrobial effect against both Gram-positive and Gram-negative bacteria. Studies on blue light inactivation of significant wound pathogenic bacteria, including Staphylococcus aureus and Pseudomonas aeruginosa, have also been reported (2,18). The action of BL on wound healing is controversial. According to studies, BL can be hazardous to several cell types, including keratinocytes, fibroblasts, retinal epithelial cells, and skin-derived endothelial cells, depending on the wavelength range and lighting circumstances. In addition, BL has a lower penetrating depth in epidermal tissue than red light (approximately 1 mm) (19). In contrast, studies point toward the fact that blue light does not impair wound healing in vitro (20). Light therapy by blue LED improves wound healing and has an antibacterial effect (21).

The study aims to evaluate the effect of 420 nm blue light LED in the context of antimicrobial blue light (aBL) phototherapy technique for treatment of induced Pseudomonas aeruginosa infected open wounds in mice.

Materials and methods

Ethical approve

The study was performed under the Ethical Standards Approved by the scientific board of College of Veterinary Medicine, University of Al-Qadisiyah (committee approval number 1314 in 18/10/2022).

Creation of infection

Thirty mature male mice were utilized. Under general anesthesia, using IM injection of a mixture of xylazine 10 mg /kg, BW and ketamine 80 mg/kg. BW (22), two circular (0.5 cm in diameter) full-thickness excisional skin wounds were performed on the back of each animal. In order to reduce mortality from bacteremia during the bleeding phase, wound infection was created 24 hrs. post wounding (PW) (23) by inoculating the right wounds in both groups with 0.2 mL of Pseudomonas aeruginosa bacterial suspension 2*10⁸ CFU/mL (9,24). The infection arose 72 hours after wounding.

Experimental design

Animals were divided into two equal groups (n = 15). G1 (control group), in which the infected wound (right side wound) was not treated and functioned as a positive control. G2 (treated group), in which the infected wounds (right side wound) were treated with blue light (LED) (5W high-power LED curing light by WOODPECKER (model LDE.C, broad-spectrum LED curing light SN L12B1227C) 420 nm wavelength, in energy dose of 30 J/cm² (25,26), single dose per day, for seven successive days, at a 1 cm distance from the light source, while protecting the left wounds from light exposure by covering with light impermeable black cover. The left wounds in both groups were not injected with bacteria and left untreated to act as a negative control on the same animal.

Morphometric examination

The wound area (size of wound), and the wound contraction were organized by directly measuring wound dimensions on the 7th and 14th-day post-infection (PI). The wound contraction was calculated by the following formula:

Percentage of wound contraction = (wound area on day 0 - wound area on day n/wound area on day 0 x 100) (27,28).

Histopathological evaluation

Specimens of healed skin (1 cm²) were collected after 7- and 14-days PI for histopathological evaluation after sectioning in 6µ and stained with H&E stain to assess the course of the healing process.

Microbial investigation

To evaluate the antibacterial activity of blue LED, wound swabs from the infected wounds were taken on the third- and seventh-day PI for bacterial count. Swab samples were placed in 1 mL of diluents containing a 0.1% peptone in 0.85% normal saline ratio of 1:99. The initial nutrient broth suspensions and their tenfold serial dilution (0.1 mL each) were cultured on nutrient agar (according to the pour plate method for bacterial count). Colonies were counted after a 24-hour incubation period at 37°C. by using the Quebec colony counter. The number of bacterial colonies forming units (CFU) per mL of sample was calculated by dividing the number of colonies by the dilution factor and multiplying the result by the volume of a specimen placed on liquefied agar (29).

Results

Surface area (size of wound) and wound contraction

The original wound size (day zero) in the treated and control wounds was 19.62 mm². At the seventh day PI, the size of non-treated infected wounds in G1 became more significant than the size of treated infected wounds with blue LED light in G2, where the size of infected wounds in G1 129.35, and 46.38% in G2 and there is a significant difference at P<0.05 between G1 and G2. On the 14th day post-infection, the size of non-treated infected wounds in G1 became more significant than the size of treated infected wounds with blue LED light in G2, where the size of infected wounds in G1 174.35 and 42.38% in G2 and there is a significant difference at P<0.05 between group one and two. In G2, the percentage of wound contraction appeared more
than in G1, where the blue LED light led to a pronounced reduction in the size of infected wounds and accelerated the stage of wound contraction (Table 1). The right infected wounds, treated with blue LED light in G2 on the seventh day post-infection, were characterized by a reduction in their size and were relatively free from infection, and they became dry and coated with thin scabs. It appears similar to the non-infected right wounds relatively. The wounds appear smaller on the 14th day post-infection than on the seventh day. In G1, the size of non-treated infected wounds appeared more significant than in G2 on the 7th and 14th day post-infection (Figure 1).

**Evaluation of antibacterial activity (wound bacterial count)**

In both groups, there were a large number of bacterial colonies (uncountable) at the 3rd day PI, and the bacterial colonies number became countable only on the third dilution. At the seventh day PI, the number of bacterial colonies becomes countable in G2 from the second dilution while remaining uncountable in G1 (Figure 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>3rd day PI</th>
<th>7th day PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>G1</td>
<td>G2</td>
</tr>
<tr>
<td>First Dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount</td>
<td>Uncountable</td>
<td>Uncountable</td>
</tr>
<tr>
<td>Second Dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount</td>
<td>Uncountable</td>
<td>Uncountable</td>
</tr>
<tr>
<td>Third Dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount (CFU/mL)</td>
<td>146 X 10^-4</td>
<td>117 X 10^-4</td>
</tr>
</tbody>
</table>

Figure 2: Antibacterial activity (CFU/mL) of blue light (LED) irradiation against induced open infected wounds by *Pseudomonas aeruginosa*, after three days and seven days PI (G1=Control), (G2=LED treated).

**Non-infected wounds in G1**

The histopathological feature of non-infected wounds (negative control) at the site of injury on the seventh-day post-infection (PI) was characterized by complete reepithelization (presence of all layers of the epidermis), thickening of the epithelial layer at the wound’s periphery, and no scab was identified. The dermis had dense immature collagen fibers and fibroblasts (Figure 3). At the same time, on the 14th day PI, the changes revealed complete re-epithelization of the epidermis, mature collagen fibers oriented parallel to the epitheliu, and myofibroblasts in the dermis, as well as the existence of discrete epithelial cells in the regenerated dermis layer (Figure 4).

**Infected non-treated wounds in G1**

The histopathological sections of infected non-treated wounds on 7th day PI were characterized by absence of epithelial layers, presence of suppurrative exudate, dead liquefied tissue, a large number of dead and live polymorph nuclear cells (neutrophils) at the center of the wound, and collagen fibers at the wound’s periphery near the intact tissue.
(Figure 5). In contrast, at 14th day the changes were characterized by absence of epithelial layers and lack signals of wound healing. The dermis had a homogenized field of dead and living neutrophils (PMNC) and debris tissue. The wound's periphery, next to healthy tissue, showed collagen fibers organized unevenly and freshly created blood vessels (Figure 6).

Table 1: Surface area and wound contraction

<table>
<thead>
<tr>
<th>Periods</th>
<th>Groups</th>
<th>Surface area (M±SE mm²)</th>
<th>Wound contraction %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>0 day</td>
<td>G1, G2</td>
<td>19.62Aa</td>
<td>19.62Aa</td>
</tr>
<tr>
<td>7 days</td>
<td>G1</td>
<td>25.38±2.88Ba</td>
<td>10.2±3.14Bb</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>9.1±3.04Ca</td>
<td>7.13±2.69Cb</td>
</tr>
<tr>
<td>14 days</td>
<td>G1</td>
<td>34.27±14.65Ea</td>
<td>8.12±4.12Cb</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>8.36±1.30Da</td>
<td>7.03±2.69Ca</td>
</tr>
<tr>
<td>LSD</td>
<td></td>
<td>1.342</td>
<td>3.018</td>
</tr>
</tbody>
</table>

-Capital letters refer to the vertical statistical comparison, whereas small letters refer to the horizontal statistical comparison.
-Different letters denote the significant difference at P<0.05, whereas similar letters refer to the no significant difference.

**Infected and treated wounds in G2**

The histopathological sections of infected and treated wounds at the site of injury on the 7th day PI were characterized by signs of infection withdrawal, complete epithelialization of epidermal layers with thin or worthy thick epidermis, and multi-orientated immature granulation tissue (Figure 7). In contrast, at 14th day PI the changes were characterized by elimination of infection, good epithelialization of epidermis, mild appearance of inflammatory cells, existence of immature granulation tissue, and abundant fibroblast attendance (Figure 8).

![Figure 3](image3.png)

Figure 3: G1; non-infected wound ten days PW (7th day PI), shows complete re-epithelization of the epidermis (EP), thickening of the epithelium at the periphery of the wound, and presence of immature dense collagen fibers (G) and fibroblasts (black arrows) in the dermis, H&E, 10X.

![Figure 4](image4.png)

Figure 4: G1; non-infected wound 17 days PW (14th day PI), shows regenerated thick epidermis (EP), dense mature collagen fibers (G) fibroblasts (black arrows), and myofibroblasts in the dermis, H&E, 10X. Black box H&E, 20X.

![Figure 5](image5.png)

Figure 5: G1, infected wound seven days PI, shows the absence of epithelial layers, suppurative exudate (SE), dead liquefied tissue, plenty of dead and live neutrophils (black arrow) in the center of the wound, and collagen fibers (G) at the periphery of the wound near the entire tissue. H&E, 10X.
Discussion

Light-emitting diodes (LEDs) are one of the three primary sources of light (Laser, LED, gas-discharge lamps) used in the field of antimicrobial photodynamic therapy (APDT). The hypothesis behind APDT’s antimicrobial activity is that when visible light activates a non-toxic chemical called a photosensitizer (PS), reactive oxygen species (ROS) are produced, which kill bacteria by inducing an oxidative rupture (30). Antibiotic-resistant bacteria cause danger to lives. APDT is a challenging technique for lowering antibiotic usage and limiting antibiotic resistance gene transfer. APDT may be considered a potential therapy among them. No injury is identified in non-photosensitized cells or cells not exposed to light. APDT can also be used safely and effectively without causing tissue damage. LEDs have become popular as lighting sources due to their ease of use, safety, and low cost (31-33).

*Pseudomonas aeruginosa* is an opportunistic bacterium that may develop biofilm, is resistant to a variety of medications, and is capable of causing harmful nosocomial infections. The recently developed antimicrobial Blue Light treatment is the most promising light-based anti-*Pseudomonas* strategy (34). Blue light in particular in the wavelength range of 405-470 nm, may cause bacterial cell death of critical wound pathogenic bacteria *Pseudomonas aeruginosa* by photo-excitation blue light sensitive chromophores, such as flavin's and cytochromes, within mitochondria or/and peroxisomes (31-37). *P. aeruginosa* infection in the mouse model was entirely eradicated by 48 J/cm² of antimicrobial aBL alone without including any additional photosensitizer molecules (38).

Results of the present study show an uncountable number of bacterial colonies in the first dilution on the third day PI (First day of treatment) in all treated wounds, where it becomes countable only on the third dilution. At day 7 PI, the number of bacterial colonies of G2 is reduced and becomes countable in the second dilution. The result shows that the blue LED used in this study has antibacterial properties against *Pseudomonas aeruginosa*. This result agrees with previous studies Amin (38) that suggested that blue light inactivation of *Pseudomonas aeruginosa* by photoexcitation of endogenous porphyrins without adding any photosensitizer molecules. *Pseudomonas aeruginosa* treated with blue light exhibits potent bactericidal activity and the inactivation of virulence factors (39). Current results also agree with Martegani (34), recognizing that the light between 410 and 455 nm displayed some anti-*Pseudomonas* action. Additionally, light at 410 nm changed the structure of ortho-nitrophenyl-D-galactopyranoside and the conformation of plasmid DNA, as well as negatively impacted the activity of the enzymes -galactosidase and catalase A. This demonstrates the potential benefit of blue light as anti-infective, and disinfection uses (34). Dai et al. (18) also looked into the possibility that the formation of
vacuoles within the cytoplasm was the first sign of blue light-mediated damage to *P. aeruginosa* cells, suggesting that this damage was related to the intracellular chromophores activated by the blue light. The antioxidant catalase can operate as a broad-spectrum target of blue light, lowering its activity through structural inactivation and causing broad-spectrum ROS sensitivity among bacteria (40).

In the present study, the blue light LED treated wounds in G2 at seven days PI realized eradication of infection and epithelialization, and the wound became smaller than the first day of infection. Wounds are seen dry, covered in a thin scab, and appear the same size as the control wound of that time. At 14 days, PI more advanced in epithelialization of treated (right) wounds are seen, and the wounds become smaller. The size was reduced by more than 50% from the original size at seven days PI, and the size reduction reached 42.66% at 14 days PI. This result, in agreement with Dai (18), catches blue light and saves mice from possibly deadly *Pseudomonas aeruginosa* burn infection. The current results prove the acceleration of wound healing. This result is supported by Adamskaya (21), who found that five days of 50 mW/cm² for 10 minutes of blue light LED at 470 nm substantially impacts wound healing. Furthermore, light treatment can benefit normal trophic wound healing by influencing keratin expression.

In G1, no signs of healing were seen in infected and non-treated wounds (infected control). The wound sizes become more significant than the original size at 7 and 14 days. PI reached 174.66%, with no wound contraction. The inoculated wounds exhibit pus discharging at seven days PI, and the pus worsens at 14 days PI. This result is according to Dakhal (41); they found an increase in the size of the wound, the presence of pus, and exaggeration of infection after induced infection with *Pseudomonas aeruginosa*. Infections with bacteria are harmful to wound healing, significantly if the wound has already delayed wound healing (42). Bacterial infections slow wound healing by prolonging the inflammatory phase (43). *P. aeruginosa* infections can cause cutaneous wounds to become chronic by preventing wound healing (44).

Numerous studies demonstrated that PDT of infected wounds significantly reduced bacterial numbers and wound size, respectively. The majority said PDT had positive effects on these characteristics. PDT helped animals with wounds infected with bacteria heal more quickly by encouraging wound closure and destroying bacteria (45). A 410 nm LED light caused both sterilization and acceleration of wound healing in mice with *Pseudomonas aeruginosa*-infected cutaneous ulcers (46). *Pseudomonas aeruginosa* was efficiently eliminated by aBL through various mechanisms, including alterations in the ultrastructure of all bacteria, endogenous porphyrins, ROS generation in bacteria, protein carbonylation, lipid peroxidation, and membrane permeability. *P. aeruginosa* showed the highest degree of endogenous porphyrins and ROS generation, making it more vulnerable to aBL (LD99.9 = 54.7 J/cm²) (47).

**Conclusion**

Blue LED offers a simple, risk-free, and affordable method of treating infected wounds. It eradicates *Pseudomonas aeruginosa* infection and enhances open-skin wound healing in mice.

**Acknowledgment**

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**Conflict of interest**

The authors declare no conflict of interest regarding the current study.

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علاج الجروح الخمجة باستخدام العلاج الضوئي بالضوء الأزرق المضاد للميكروبات

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الخلاصة

هدفت الدراسة إلى التحقق من تأثير الضوء الأزرق في علاج الجروح الخمجة، استخدم ثلاثين فأرا ذكر قسمت إلى مجموعتين المجموعة الأولى (السيطرة) والمجموعة الثانية (المعالجة). تم عمل جريحين دائريين (0.5 سم) على ظهر الحيوان، وتم إحداث الخمج في الجرح الأيمن بعد 24 ساعة عن طريق حقن 0.2 مل من معلق بكتيريا الزوائف الزنجارية، وترك الجرح الأيسر بدون حقن كعنصر سيطرة في نفس الحيوان. الجروح الخمجة في المجموعة الأولى لم تعالج، ووصفت الجروح الخمجة في المجموعة الثانية بالضوء الأزرق 420 نانومتر 30 جول/سم²، جرعة واحدة / يوم، لمدة 7 أيام متتالية. تم تقييم الشفاء عن طريق قياس حجم الجروح، والعد البكتيري، والخزعات النسيجية المرضية المأخوذة عند 7 و 14 يوما بعد الخمج. أوضحت النتائج أن حجم الجروح الخمجة في المجموعة الأولى أصبح أكبر معنويًا 174.66 و 174.66% في 7 و 14 يوما بعد الخمج على التوالي. وفي المجموعة الثانية تم القضاء على الخمج في الجروح المعالجة وانخفض حجم الجروح معنويًا 46.38 و 42.66% في 7 و 14 يوما على التوالي. نسبياً مرضياً. أظهرت الجروح الخمجة في المجموعة الأولى عند 7 و 14 يوم وجود نضحة قيحيه، وانسجة ميتة، مع وجود العدالات الميتة والحيية. وأظهرت الجروح المعالجة للمجموعة الثانية في 7 أيام، تكون ظاهرة البشرة، مع وجود الأنسجة الحبيبية غير الناضجة. في 14 يوما كانت البشرة متجددة بشكل جيد، مع وجود ألياف الكولاجين الناضجة في الأدمة. تستنتج من الرسالة الحالية أن علاج الضوء الأزرق يقضي بشكل فعال على الخمج بالزواائف الزنجارية ويساعد في الشفاء.