Effect of blue laser on viability of *Proteus mirabilis*

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Abstract:
The usage of blue laser has been considered as a therapeutic approach to prohibit the viability of bacterial species, but there is no agreement about optimum parameters to be used. The aim of this project is to study the influence of blue laser (450 nm) on the viability of the gram-negative bacteria *Proteus mirabilis* isolated from burn wounds, using different exposure times (i.e., doses) in vitro. Seventy swab samples were collected from burn wounds of patients admitted to the burns unit in AL-Yarmouk teaching hospital in Baghdad, during the period from June to August 2019. The Bacteria were isolated and identified depending on their culture characteristics, biochemical tests, gram staining, and morphology, being finally confirmed by API 20E Test System. By using the disk diffusion method, susceptibility of the isolates to 12 different antibiotics was examined. One isolate of *P. mirabilis* was elected according to susceptibility to all antibiotics used. To prepare bacterial solution, *P. mirabilis* was mixed with normal saline solution. Dilution of 10^6 cell/ml for *p. mirabilis* was selected from other serial dilutions. A number of colonies and colony forming units (CFUs/ml) were achieved and correlated to controls. *P. mirabilis* was irradiated by blue diode laser (450 nm, 500mw) and exposed to different doses (24, 48, 72, 96, 120J/cm²) corresponding to respective exposure times (4, 8, 12, 16, 20 minutes). The results of antibiotic susceptibility test indicate that the entire isolates of *P. mirabilis* were multidrug resistant. With the increase in laser dose (exposure times), the number of colonies and CFUs/ml were reduced, reaching a highest inhibition in CFU/ml at exposure time of 20 minutes, i.e., a dose of 120J/cm², with irradiance of 0.1 watt/cm². No significant reduction was recorded in CFU/ml at exposure time of 4 min (a dose of 24J/cm²). As a conclusion, the blue laser irradiation at wavelength of 450 nm and 500nm had antibacterial effects on *P. mirabilis* isolated from burn wounds with irradiance of 0.1 watt/cm² in vitro, as evidenced by the effective reduction in the viability of bacteria at a dose of 120J/cm² corresponding to exposure time of 20 minutes.

Keywords: blue laser, proteus mirabilis, phototherapy.

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albicans is a member of Enterobacteriaceae family of bacilli. It is a gram-negative bacterium with inability to ferment lactose and ability to ferment maltose. When *P. mirabilis* contacts with solid surfaces, it has the ability to secrete polysaccharides and self-elongate, which permits an action of obvious motility along surfaces of medical apparatus. It also has strong motility, where the flagella of *proteus mirabilis* are responsible for its motility that is correlated with the capability to secrete biofilms. This motility is utilized to support colonization and implied to contribute to resistance to definitive antibiotics [1-3].

*P. mirabilis* is associated with infections of ulcers and wounds [4-7]. Over years, the bacterium has been recognized as a common pathogen isolated from infected wounds. Furthermore, it expresses multidrug resistance (MDR), which evolved to represent an essential clinical issue. Therefore, there is a necessity to develop strategies that counteract the increase of MDR [8].

Following the invention of laser in the past century, there was continuous development in applying laser radiation on microorganisms and biological tissues. In recent years, the visible light has been employed to investigate its bactericidal influence, and the blue part (430-480nm) has been concluded to be effective in killing different microorganisms [9-12]. Kazemikhoo et al. [13] used low intensity laser in the treatment of burn wounds and cutaneous ulcers to enhance the process of healing. Another *in vitro* study focused on using laser radiation of 425 nm, which showed bactericidal influences on the growth of *Staphylococcus aureus* and *Escherichia coli* [14].

The effects of laser radiation and its associated parameters, such as wavelength, irradiance, and dose, on the growth of microorganisms are inadequately understood. A number of previous studies demonstrated that laser effects on bacteria were strongly dependent on wavelength, irradiance, and species, which are most important parameters for prohibiting bacterial growth [15-17].

Since there are no studies that describe the influence of blue laser on *P. mirabilis* growth, the aim of the current study is to examine the effects of blue laser on the viability of *P. mirabilis* isolated from burn wounds in *vitro* at different exposure times (doses).

**Methods**

**Isolation and identification of bacteria**

Through the period extending from June to August 2019, seventy swab samples were collected from burn patients (burn wounds) from the burns unit at Al-Yarmouk Teaching Hospital in Baghdad.
using sterile cotton swabs in transport media. Patients on antibiotic treatment were eliminated from the study. Swabs were obtained from areas that showed deep-seated wounds with discharge. Isolation and biochemical analysis of Proteus mirabilis were performed at Al-Yarmouk Teaching Hospital Laboratories. Burn wound swabs were directly streaked on blood and macconkey agars and then incubated aerobically for 24 hours at 37°C. Identification of the isolates was accomplished depending on the biochemical and culture characteristics, according to the API 20E Test System [18].

**Antibiotic susceptibility test**

Kirby–Bauer method was used, as reported by the laboratory standards institute [19], to achieve the antibiotic susceptibility test for 12 different antibiotics that are commonly used to treat burn wounds: Gentamicin, Ampicillin, Ampiclox, Amikacin, Tetracycline, Erythromycin, Ciprofloxacin, Imipenem, Meropenem, Cefotaxime, Ceftazidime, and Trimethoprim/sulphamethoxazole. The isolates were classified as resistant, intermediate, or sensitive to antibiotics according to the values obtained by the National Committee for Clinical Laboratory (NCLS) [20].

**Blue laser experiments**

A portable continuous wave diode laser (UK -scientific Ltd) was used in all experiments of wavelength (450 nm , 500 mw). The treatment parameters of laser used in this study are shown in table 1. A readjustment of laser equipment was made at the beginning of each experiment to verify precise doses during the experiments. A laser power meter (SoLo PEgenetic -Eoinc Canada) was used to measure the output power of the laser used.

**Table 1- Treatment parameters of blue laser used in this study.**

| Parameter                                      | Specification |
|------------------------------------------------|---------------|
| Wavelength                                     | 450nm         |
| Output power of laser ( mw)                    | 500 mw        |
| Spot size of laser beam ( cm²)                 | 5cm²          |
| Irradiance measured at target area ( watt/cm²)| 0.1watt/cm²   |
| Dose= Irradiance (watt/cm²) × exposure time.  |
| Dose= (watt × sec)/ cm²                        |
| Dose= J/ cm²                                   |
| Exposure time( min)                            | 4   8  12  16  20 |
| Dose J/cm²                                     | 24  48 72 96 120 |

The laser was fitted with a convex lens which is used as a beam expander at the end to illuminate a circular area. Laser spot size of 5 cm² was produced by using a convex lens that is keeping the distance between the microtiter plate and the lens definitive. The value of irradiance measured at the 5cm² target area (microtiter plate) was 0.1 watt/cm².

One isolate of *P. mirabilis* was elected according to virulence factors, including urease production, adherence capability, having flagella, and susceptibility to all antibiotics used [21]. To prepare the bacterial solution, the selected isolate was cultured on Macconkey agar, from which few colonies were transferred and mixed with sterile saline solution. The turbidity of bacterial suspension was determined to an optical density of 0.5 by using spectrophotometer at a wavelength of 530 nm. After that, serial dilutions were prepared, from $10^5$ to $10^{10}$, and the dilution of $10^6$ was selected, which contained 30-300 colonies [22]. Four millilitres of bacterial suspension, with $10^6$ cell/ml, was placed in a sterilized test tube and kept unirradiated by laser (control group). By using L-shape spreader, an
aliquot of 100μl out of 4ml (bacterial dilution of 10^6 cell/ml) was spread on nutrient agar. Eight plates were prepared as control plates for each assay and incubated aerobically at 37 °C for 24 hours, and then CFU/ml was calculated after incubation (control group). Another four millilitres of bacterial suspension, with 10^6 cell/ml, was placed in wells of microtiter plate, distributed at an area of 5cm² in accordance with laser spot size of 5cm² and exposed to different doses (24, 48, 72, 96, 120 J/cm²) of blue laser, corresponding to 4.8,12,16,20 minutes. After irradiation by laser light, 100μl out of 4ml bacterial suspension, with 10^6 cell/ml, was spread on nutrient agar using L-shape spreader. Eight plates were prepared for each assay and incubated aerobically at 37 °C for 24 hours (Irradiated groups) and CFU/ml was then calculated. Manual colony counter (colony counter, SC6, Fisher scientific) was used to count bacterial colonies on agar plate.

For each experiment, bacterial suspension was freshly prepared and each experiment had its control plates, to reduce the variability. All apparatus were adjusted at the beginning of the study to confirm that they delivered definite doses at the time of each experiment. The method of irradiation was systematized before the experiments. To improve data precision, each experiment for the irradiated dose was repeated 3 times. CFU is a measure of viable cells of bacteria. CFU/ml can be calculated using the following formula [22]:

\[
\frac{CFU}{ml} = (\text{Colony number}) \times \text{dilution factor} \times \frac{1}{\text{volume plated}}
\]

Volume plated = 100μl = 0.1 ml

Statistical analysis

Statistical analysis was achieved by using the statistical package for social sciences (SPSS, version 20.0 for windows, SPSS, Chicago, IL, USA). Data were presented as mean ± standard deviation SD (95% Confidence Interval). Data normal distribution was examined using the Shapiro-Wilk test, where colony number and CFU were not normally distributed (0.885, P=0.004). Comparisons between control and irradiated groups were performed using the Wilcoxon signed ranks test. Comparisons between irradiation times were conducted using the Kruskal-Wallius test. Statistically significant differences were considered at p<0.05.

Results

Bacterial isolates

The outcomes of isolation and identification of bacteria showed that 70 different isolates from burn wounds (25.714 %, 18 isolates) were P. mirabilis. Each isolate was tested for susceptibility to 12 different disks of antimicrobial agents. The outcomes exhibited that all the isolates of P. mirabilis were resistant to 4 antibiotics, namely Ampiclox, Ampicillin, Tetracycline, and Erythromycin (table 2), while they showed lower resistance for Gentamicin (77.77%), Ciprofloxacin (77.77%), Cefotaxime (65%), Meropenem (61%), Ceftazidine (60%), Amikacin (50%), Trimethoprim/ sulphamethoxazole (44%), and Imipenem (38%) (Table 2).

Table 2- Percentage of resistance of P. mirabilis against various types of antimicrobial agents.

| Microbial agents                  | No of all isolates | Resistance | Sensitive | Intermediate |
|-----------------------------------|--------------------|------------|-----------|--------------|
| Gentamicin (30 μg)                | 18                 | 14         | 77.77     | 3 16.66 1 5.5 |
| Ampicillin (30 μg)                | 18                 | 18         | 100       | - - - -     |
| Ampiclox (10 μg)                  | 18                 | 18         | 100       | - - - -     |
| Amikacin (30 μg)                  | 18                 | 9          | 50        | 7 38.88 2 11.11 |
| Tetracycline (10 μg)              | 18                 | 18         | 100       | - - - -     |
| Erythromycin (10 μg)              | 18                 | 18         | 100       | - - - -     |
| Ciprofloxacin (10 μg)             | 18                 | 14         | 77.77     | - - 4 22.22 |
| Imipenem (10 μg)                  | 18                 | 7          | 38.88     | 8 44.44 3 16.66 |
| Meropenem (10 μg)                 | 18                 | 11         | 61.11     | 5 27.77 2 11.11 |
| Cefotaxime (30 μg)                | 18                 | 12         | 65        | 6 35 - -   |
| Ceftazidine (30 μg)               | 18                 | 11         | 60        | 7 40 - -   |
| Trimethoprim/ sulphamethoxazole. (10 μg) | 18 | 8 | 44.44 | 6 33.33 4 22.22 |
The influence of laser radiation on viability of *P. mirabilis*

The results of the influence of blue laser on colony number (colony count) of *P. mirabilis* at irradiance of 0.1 watt/cm² and various exposure times are shown in Table 3. The mean colony number and viability of *P. mirabilis* (CFU/ml) were significantly reduced at exposure times of 8, 12, 16, and 20 min (doses 48, 72, 96, 120 J/cm², respectively) as compared with control groups (Tables 3 and 4). However, effective reductions in colony number and CFU/ml of *P. mirabilis* were obtained after 20 min, i.e. dose of 120 J/cm² (Tables 3 and 4).

Figure 1 shows the relationships of different exposure times of laser (4, 8, 12, 16, and 20 minutes) corresponding to their respective doses (24, 48, 72, 96, and 120 J/cm²) on viability of *P. mirabilis* (CFU/ml). Wavelength of 450 nm laser radiation produced a statistically significant decline in the colony number and CFU/ml, which was parallel to increasing the dose (Tables 3 and 4; figure 1).

Table 3- Mean and standard deviation values of colony number (colony count) of *P. mirabilis* irradiated by 450 nm laser light at irradiance of 0.1 watt/cm² and different exposure times (doses).

| Exposure time (min) | Dose J/cm² | Control colony number | Irradiated colony number |
|--------------------|------------|-----------------------|-------------------------|
|                    |            | Mean±SD               | CI 5-95%                | Mean±SD               | CI 5-95%                | P value   |
| 4                  | 24 a       | 105±35                | 69-159                  | 106±36                | 77-166                  | 0.893     |
| 8                  | 48 b       | 88±15                 | 67-109                  | 65±11                 | 48-75                   | 0.043*    |
| 12                 | 72 c       | 106±9                 | 96-117                  | 57±7                  | 49-64                   | 0.043*    |
| 16                 | 96 d       | 111±10                | 98-120                  | 25±4                  | 21-30                   | 0.043**   |
| 20                 | 120        | 102±13                | 88-121                  | 6±2                   | 4-7                     | 0.043*    |

Dose (J/cm²) = Irradiance (watt/cm²) × exposure time.

* = significant difference between control and irradiated groups using Wilcoxon Signed Ranks test.

Comparing irradiated colony numbers at different irradiation times using Kruskal-Wallis test (X²=27.473, P<0.005):

a: significant difference between 24 J/cm² and 48, 72, 96, and 120 J/cm² irradiation (P<0.005).
b: significant difference between 48 and 96 J/cm², and 120 J/cm² irradiation (P<0.005).
c: significant difference between 72 J/cm² and 96 J/cm², and 120 J/cm² irradiation (P<0.005).
d: significant difference between 96 J/cm² and 120 J/cm² irradiation (P<0.005).

Table 4 - Mean and standard deviation values of viability of *P. mirabilis* CFU/ml irradiated by 450 nm laser light at irradiance of 0.1 watt/cm² and different doses.

| Exposure Time (min) | Dose J/cm² | Control CFU/ml | Irradiated CFU/ml |
|---------------------|------------|----------------|-------------------|
|                     |            | Mean          | SD                | CI 5-95%            | Mean           | SD                | CI 5-95%            | P value   |
| 4                   | 24 a       | 1.048X10⁶      | 353X10⁶           | 690X10⁶-1.590X10⁶   | 1.056X10⁶     | 356X10⁶           | 770X10⁶-1.660X10⁶   | 0.893     |
| 8                   | 48 b       | 882X10⁶       | 148X10⁶           | 670X10⁶-1.090X10⁶   | 648X10⁶      | 107X10⁶           | 480X10⁶-750X10⁶     | 0.043*    |
| 12                  | 72 c       | 1.058X10⁶     | 92X10⁶            | 960X10⁶-1.170X10⁶   | 574X10⁶      | 73X10⁶            | 490X10⁶-640X10⁶     | 0.043*    |
| 16                  | 96 d       | 898X10⁶       | 443X10⁶           | 120X10⁶-1.180X10⁶   | 248X10⁶      | 35X10⁶            | 210X10⁶-300X10⁶     | 0.080*    |
| 20                  | 120        | 1.024X10⁶     | 128X10⁶           | 880X10⁶-1.210X10⁶   | 56X10⁶       | 15X10⁶            | 40X10⁶-70X10⁶       | 0.043*    |

Dose (J/cm²) = Irradiance (watt/cm²) × exposure time.
** = significant difference between control and irradiated groups using Wilcoxon Signed Ranks test.

Comparing CFU values at different irradiation times using Kruskal-Wallis test ($X^2=27.473$, $P<0.005$):

a: significant difference between 24 J/cm$^2$ and 48, 72, 96, and 120 J/cm$^2$ irradiation ($P<0.005$).

b: significant difference between 48 J/cm$^2$ and 96 and 120 J/cm$^2$ irradiation ($P<0.005$).

c: significant difference between 72 and 96, and 120 J/cm$^2$ irradiation ($P<0.005$).

d: significant difference between 96 J/cm$^2$ and 120 J/cm$^2$ irradiation ($P<0.005$).

Figure 1 - The relation between blue laser radiation at different exposure times and viability of *P. mirabilis* (the mean of CFU/ml with its 95% confidence interval). $Dose = Irradiance (watt/cm^2) \times$ exposure time.

**Discussion**

The results of this study showed that the blue laser at a wavelength of 450 nm had a bactericidal effect on the multidrug resistant *P. mirabilis* bacteria at exposure times of 8, 12, 16, and 20 minutes, corresponding to doses of 48, 72, 96, and 120 J/cm$^2$), at irradiance of 0.1 watt/cm$^2$. These results are in accordance to an earlier study conducted by Rupel et al. [23] who found that the blue laser light at a wavelength of 445 nm had a bactericidal effect on gram negative bacteria *Pseudomonas aeruginosa* in planktonic growth at doses of 40, 60, and 120 J/cm$^2$ and irradiance $\leq 0.30$ watt/cm$^2$. In the present study, the inhibiting rate of *P. mirabilis* started at a dose of 48J/cm$^2$, which is relatively compatible with the results of Enwemeka et al. [24], who used blue laser (150mw, 470nm) at various doses (1,3,5,9,7,13,11,15,17,19,25,35,30,40,45,50,55,60 J/cm$^2$) to determine the bacterial inhibition of two strains of methicillin-resistant *S. aureus* (MRSA). Irradiation with blue laser produced a statistically significant dose-dependent inhibition in the number of colonies formed by the two strains. More bacterial inhibition was achieved as the dose was increased, but the influence was non-linear and better notable at lower doses. Also, the results of Enwemeka [25] are consistent with those of the present study, as he noticed that using 405nm laser (500mw) can prohibit the viability of two strains of MRSA at the same doses used in his aforementioned study [24]. He concluded that blue laser irradiation resulted in a statistically significant dose–dependent inhibition in the number of colonies in both strains of MRSA; as irradiation time was increased the viability of bacteria was decreased.
Another study by Guffey and Wilborn [26] demonstrated that exposure to 407 nm laser light completely killed *P. aeruginosa* at doses of 1, 3, 5, 10, and 15 J/cm², but inhibited *S. aureus* at 10 and 15 J/cm². The 405 nm light at doses of 1, 3, 5, 10, and 15 J/cm² provided a dose-dependent antimicrobial influence on *S. aureus* and *P. aeruginosa*. de Sousa and co-workers [27] demonstrated that the blue light had an inhibition effect on the growth of *P. aeruginosa*, *Escherichia coli*, and *S. saureus* at doses higher than 6 J/cm², but *Escherichia coli* was inhibited at all doses used, except the dose of 24 J/cm². In the current study, the maximum inhibition of *P. mirabilis* was achieved at the dose of 120 J/cm². However, the results of the present and the previous studies support the assumption that irradiation parameters (output power of laser, irradiance, exposure time) can affect the results definitely [23-29].

The optimum effect of blue laser on *P. mirabilis* has not been yet fully investigated, and more studies are needed to determine the irradiation parameters used to inhibit this pathogenic bacterium. The bactericidal impact of the blue light may be explained according to the outcomes of a previous study by Yin *et al.* [30], which showed that higher doses of light with wavelengths longer than 400 nm are capable of destroying bacteria. Treatment with UV kills gram negative and positive bacteria due to the absorption of the energy of light by cytosine and thymidine, the pyrimidine bases of DNA. The energy absorbed separates the bonds and allow the UV radiation to alter the base which will communicate with adjacent bases through modifying the architectural arrangement. The irradiation dose necessary for killing cells is achieved when the rate of DNA destruction is increased to limits above the rate of repair [31]. It is apparent that photo-destruction of DNA by the blue light causes antibacterial effects on *P. mirabilis* and other gram positive and negative bacteria [32-36]. More researches are needed to understand the actual mechanisms concerned with the influences of blue light on bacteria. As shown in tables 3 and 4, *P. mirabilis* was inhibited at doses of 48, 72, 96, and 120 J/cm². These significant inactivations of bacteria at these doses indicate that laser light at 450 nm with irradiance of 0.1 watts/cm² may be a practical alternative to treatment with antibiotics, particularly in subcutaneous and cutaneous *P. mirabilis* infections. The possible clinical application of these outcomes can be achieved by the transmission of laser light through the structure of a probe with handle use. This design would perfectly adjust to an open wound infection.

**Conclusion**

The results demonstrate that *in vitro* 450 nm blue laser light at doses of 48, 72, 96, and 120 J/cm² produces a bactericidal influence on *P. mirabilis* isolated from infected burn wounds. This approach of killing *P. mirabilis* may suggest original method to heal wounds infected by this colonized bacteria.

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