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Antibacterial Photodynamic Effect of 532 nm Diode-Pumped Solid State and 650 nm Diode Lasers on Methicillin Resistant *Staphylococcus Aureus* in Vitro

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Abstract: The photodynamic inactivation against Methicillin-resistant *Staphylococcus aureus* using two different lasers, 532 nm diode pumped solid state laser (DPSS) in combination with safranin O and 650 nm diode laser in combination with methylene blue was investigated in the present work. A hundred swab samples were collected from patients with burn and wound infections admitted to two hospitals in Baghdad (Specialized Burns Hospital in Medical City and Al Imamein Al Jwadein Medical City Hospital) from December 2015 to February 2016 Antimicrobial susceptibility was performed by using Kirby- Bauer method. The irradiation experiments included four groups; a control group, a photosensitizer only group, a laser irradiation only group and a laser irradiation combined with a photosensitizer group. The results showed that 532nm DPSS laser with power density 0.157 W/cm^2 combined with 0.5 mg/ml safranin O was more effective than 650 nm diode laser with power density 0.052 W/cm^2 combined with 0.1 mg/ml methylene blue in reducing the number of MRSA cells. One hundred percent killing of MRSA was achieved after 3 minutes exposure to 532 nm DPSS laser in combination with safranin O, while it took 11 minutes to achieve the same result using 650 nm diode laser and methylene blue. In conclusion, photodynamic inactivation can be considered as an alternative method in treating superficial burn wound infections.

Keywords: *Laser, Methicillin-resistant Staphylococcus aureus, Safranin O, Methylene blue*

Introduction

Staphylococcus aureus is one of the most important pathogens isolated from burn and wounds infections and responsible for serious complications following damage of human skin (Church *et al.*, 2006). In the case of burn and wound infection, surgical treatment usually is carried out with the use of antibiotics and antiseptics as accompaniment therapies. Nevertheless, long-term use of these agents can be rendered ineffective by resistance developing in the target organism (Garcez, 2010). Accordingly, overuse of antibiotics for treating skin infections worldwide has increased the bacterial resistance to a greater number of antimicrobial agents (Lowy, 2003). In the United States, MRSA was first reported in 1968 and has become a widely recognized cause of morbidity and mortality throughout the world (Raygada *et al.*, 2009). Since the primary report on laser radiation by Maiman in1960, numerous potential fields for its application have been explored. Various kinds of lasers have already become irreplaceable tools of modern medicine (Niemz, 2007). Photodynamic therapy (PDT) is a treatment which uses a combination of a drug (harmless dyes), called photosensitizer and a particular type of visible light that, in the presence of oxygen, produces reactive oxygen species that damage biomolecules and kill cells (Gomer, 2010). The nature of photodynamic therapy makes it ideal for the treatment of skin

wound and burn infections, all of which are easily accessible for light therapies (Sharma *et al.*, 2011). The aim of the present study is to evaluate the combined effect of 532 nm DPSS laser with safranin O and 650 nm laser with methylene blue on the growth of MRSA.

Materials and Methods

A hundred swap samples were collected from skin burn and wound areas using sterile disposable swabs in transport media. These swaps were taken from patients admitted to burn unit in Medical City hospital and Al-Kadhimiya Teaching Hospital in Baghdad during the period from December 2015 to February 2016. The samples were cultured on mannitol Salt agar and incubated aerobically at 37°C for 24 hours. The colonies were purified by transferring a single pure isolated colony to brain heart infusion (BHI) agar. *S. aureus* grown on mannitol salt agar (selective medium) were identified using conventional cultural and biochemical methods (Adams, 2000; Harley and Prescott, 2002; vandepitte *et al.*, 2003) in addition to VITEK2 test for confirmation.

Antimicrobial susceptibility was performed on Mueller-Hinton agar using disk method of Kirby- Bauer (Kirby and Bauer, 1966). The antibiotics discs that were used included: Augmentin (Amoxicillin/ clavulanic Acid) (20 µg), Vancomycin (30 µg), Chloramphenicol (30µg) and Erythromycin (15 µg), Ciprofloxacin (10 µg), Methicillin (10 µg), Penicillin (10 µg) and Rifampcin (5 µg), Gentamicin (10 µg), Oxacillin (1 µg), and Tetracyclin (10 µg)

The most resistant *S. aureus* isolate to antibiotics (Augmentin, and Erythromycin, Ciprofloxacin, Methicillin, Penicillin and Rifampcin, Gentamicin, Oxacillin, and Tetracyclin) was selected and considered MRSA**.** Suspension of each bacterial growth with dilution of 10^{-5} was chosen according to preliminary trials of viability count. The experimental samples were prepared by placing 0.5 ml of the bacterial suspension in each one of two Eppendorf tubes. One tube was completed to 1 ml by adding 0.5 ml normal saline while the other one was completed to 1 ml by adding 0.5ml of photosensitizer in final concentration (0.5 mg/ml for safranin and 0.1 mg/ml for methylene blue). The samples were then subjected to laser irradiation experiment. The laser system used in the experiment was 532 nm DPSS laser with power density 0.157 W/cm^2 and 650 nm Diode laser with power density 0.052 W/cm^2. The irradiation experiment included the following four groups all of which were performed in the dark for both lasers:

Group I (L-P-): This group was considered as a negative control. It was not subjected to laser or photosensitizer.

Group II (L-P+): This group was treated with the (0.5 and 0.1) mg/ml photosensitizer only (safranin O or methylene blue). It was considered as a second control group.

Group III (L+P-): This was the one that was treated with laser radiation only without adding the photosensitizer; instead it was mixed with equal amount of saline solution.

Group IV (L+P+): This group was irradiated with laser light in the presence of photosensitizer.

The laser beam was defocused on the surface of the suspension at a distance of 21 cm, and a beam diameter of 1 cm, using a concave lens. The exposure times applied were from 0.5 minute and to 35 minutes at 2 minutes intervals. After irradiation, an amount of 100 µl of the irradiated bacterial suspension was spread evenly over the surface of Mannitol Salt Agar, and five plates were used for each experimental group. The inoculated plates were then incubated aerobically at 37 °C for 24 hrs. The number of colonies was counted using a colony counter and the colony forming units (CFUs) was calculated.

Statistical analysis

The results were log-transformed and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The P values <0.05 were considered significant. Data are presented as mean and standard deviation (S.D.).

Results and Discussion

The results of antibiotic susceptibility test showed that the highest resistant of *S. aureus* isolates were observed for Methicillin and Penicillin (100%), followed by Erythromycin (88%), Oxacillin (84%), Augmentin and Rifampin (68%), Ciprofloxclin (64%) and Gentamycin (52%) (Fig.1). By contrast, all *S. aureus* isolates (100%) were sensitive to Chlormphenicol, and less sensitive to Vancomycin (88%) Figure 1.

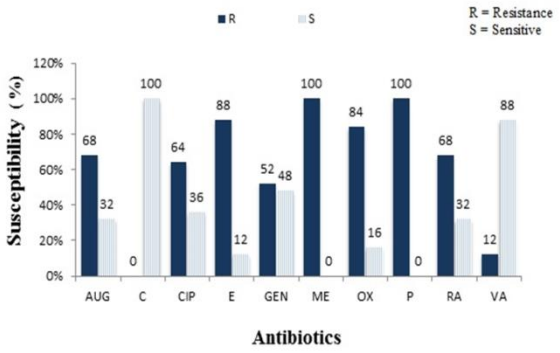

Fig. (1): The Susceptibility of S. aureus to 10 antibiotics.

The results of the identification showed that 25% of the isolates were *S. aureus.* This proportion is almost similar to the finding of other researchers. For example, Alwan (2011)**,** found that 24% of the total isolate were *S. aureus***.** Another study which was conducted by Al-Taie *et al.* (2014) in Baghdad, revealed that *S. aureus* constituted 20.2% of the bacterial isolates**.** Qader and Muhamad, (2010) reported more proportion (34%) than our findings about the patients admitted in Sulaimani Plastic and Burn hospital. This variation in the proportions of *S. aureus* in different cities may be contributed to the different geographical location and environments. The results of 532 nm laser irradiation experiments revealed that laser irradiation alone without photosensitizer (L+P–) showed no significant effect in reducing the number (log CFU/ml) of MRSA compared with the control group (L–P–) Figure 2.

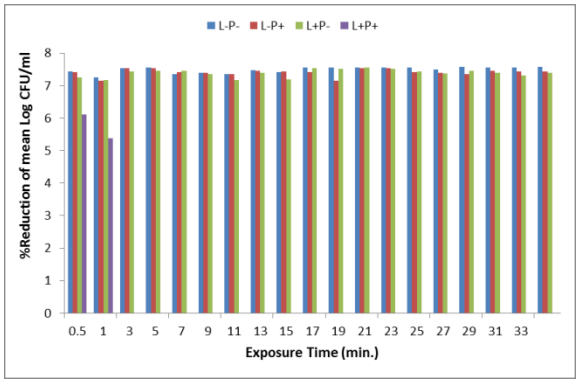

Fig. (2): The Effect of 532 nm DPSS laser with power density of 0.157 W/cm2 on the growth of MRSA at different exposure times.

The combined effect of the same laser with Safranin O significantly reduced the bacterial growth (CFU/ml) of MRSA by 95.22% and 98.64% after 0.5 min and 1 min exposure to DPSS laser irradiation respectively, and reached a remarkable complete reduction (100 %) at three minutes exposure time as shown in (Table 1).

Table 1 : The Percentage of reduction, deduced from mean values of CFU/ml, for the viability of MRSA exposed to DPSS laser in the presence of photosensitizers (L+P+) in relation to the group treated neither with laser nor with photosensitizer (L-P-) at different exposure times.

Exposure time (Min.)	diode pumped solid state 532 nm with Safranin O		
	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)
0.5	1280000	26800000	95.22
1	240000	17666667	98.64
3	0	35000000	100
5	-----	-----	-----

In the case of 650 nm Diode laser, the results showed that irradiation of MRSA with this laser alone reduced the number of Log CFU/ml significantly ($P<0.05$) at 11 minutes exposure (L+P–) compared with the control group (L–P–). The number of Log CFU/ml continued decreasing slightly with increasing the time of exposure to laser light. However, no complete mortality was reached after 35 minutes Figure 3).

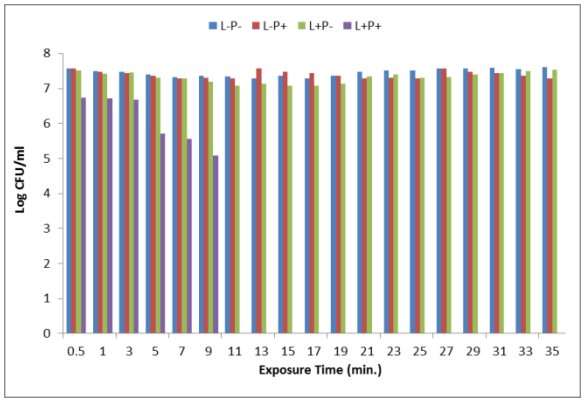

Fig. (3): The Effect of 650 nm Diode laser with 0.052 W/cm2 power density on the growth of MRSA at different exposure times.

On the other hand, the combined effect of 650 nm diode laser with photosensitizer (MB) (L+P+) showed a highly significant (P<0.001) reduction in the number of Log CFU/ml of MRSA compared with other groups (L−P−, L−P+ and L+P−) for all times employed in this experiment (0.5, 1, 3, 5, 7, 9 & 11 min) and reached a complete mortality (100%) at 11 minutes exposure time as shown in Figure 3 and Table 2.

Table (2) : The Percentage of reduction, deduced from mean values of CFU/ml, for the viability of MRSA exposed to diode laser in the presence of photosensitizers (L+P+) in relation to the group treated neither with laser nor with photosensitizer (L-P-) at different exposure times.

Exposure time (Min.)	diode laser 650 nm with Methylene blue		
	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)
0.5	5520000	37733333	85.37
1	5240000	31666667	83.45
3	4800000	2926667	83.60
5	520000	24600000	97.88
7	360000	21266667	98.31
9	120000	22666667	99.47
11	0	21800000	100

The results of viability test for MRSA using DPSS laser agrees with the results obtained by Al-Zubaidy and Maki (2015). They found that laser irradiation alone did not give a significant reduction in the number of log CFU/ml as the combination of laser light with photosensitizer (Safranin O); the latter reduced the number of log CFU/ml with increasing time of exposer and reached 100% mortality at 5 minutes exposure time**.** Another study conducted in Iran, using three different types of lasers, showed that SHG Nd:YAG laser at 532 nm combined with Safranin O slightly inhibited the growth of *S. aurue* (Dadras *et al*., 2006). This low growth inhibition may be explained by the fact that pulsed Nd:YAG laser used, had different effect on the bacteria than the CW laser used in this study (532 nm DPSS). In another study, incoherent light-emitting diode (LED) seems to have a bactericidal effect also at different wavelengths (425, 525 and 625 nm) (Kim *et al.*, 2013). They showed that the bactericidal effect was stronger at short wavelength (425 nm) than longer wavelength (625 nm), and reported 30-90% reduction in the survival of *S.aureus* at 525 nm wavelength, which is close to the wave length of our laser (532 nm).

No reports were found on the effect of 650 nm laser irradiation on *S. aureus* or MRSA. However, there are few studies on the effect of other lasers on *S. aureus* or MRSA at wavelengths close to 650 nm. Kashef *et al.*, (2012) studied the effect of 660 nm diode laser (35 mW) with two photosensitizers (Methylene blue and Toluidine blue O) on *S. aureus*. They found that irradiation by a combination of 660 nm diode laser with methylene blue reduced the number of log CFU/ml of *S. aureus* and MRSA after 30 minutes exposure (Kashef *et al.*,2012). Another investigator studied the effect of 632 nm diode laser in the presence of Methylene blue on MRSA (Ismael, 2014). He recorded a maximum decrease in viable colony counts (99% killing of cells) at 15 minutes exposure time. Another in vivo study conducted by Silva *et al.*, (2013) showed that irradiating bacteria-infected wounds in the skin of rats with 658 nm red laser diode (AlGaInP) with a dose of 5J/cm^2 reduced bacterial proliferation of MRSA. 532 nm DPSS laser showed a better bactericidal effect on MRSA than 650 nm diode laser.

DPSS 532 nm lasers are relatively superior in their effectiveness over 650 nm diode lasers. This may be attributed to better beam quality, less divergence and higher energy per photon of 532 nm DPSS lasers. Two reasons we reckon that contributes to this finding. The first the power density in case of the 532 was higher than the 650 laser. The second was because that the DPSS laser with 532 nm wavelength and 2.3 eV/photon energy per photon was superior to 650 nm diode laser with photon energy of 1.9 eV/photon.

So shorter wavelength lasers are more effective in inhibiting bacteria growth because of the fact that energy per photon increases with decreasing wavelength.

Conclusion

Staphylococcus aureus isolates showed high resistance to most antibiotics used in this study. The two lasers alone without photosensitizers used in this work (532 nm DPSS laser at power density of 0.157 W/cm^2 and 650 diode laser at power density of 0.052) were not able to give a complete eradication of MRSA at all times of exposures. DPSS laser (532 nm) irradiation combined with photosensitizer safranin O was remarkably effective in killing 100% of MRSA cells in short time (≥ 3 minutes exposure), while 650 nm diode laser combined with methylene blue was less effective than DPSS laser (11 minutes for eradication of MRSA). The shorter wave length has higher photon energy to induce more production of toxic ROS that is responsible for bacterial mortality.

References

Church, D., Elsayed, S., Reid, O., Winston, B., Lindsay, R. (2006) Burn Wound Infections. Clinical Microbiology Reviews. **19**(2): 403 – 434.

Garcez, A. S., Nuñez, S. C., Hamblim, M. R., Suzuki, H., and Ribeiro, M. S. (2010). Photodynamic Therapy Associated with Conventional Endodontic Treatment in Patients with Antibiotic-resistant Microflora: A Preliminary Report. American Association of Endodontists. **36**(9): 1463-6.

Lowy, F.D. (2003). Antimicrobial resistance: the example of Staphylococcus aureus. J. Clin. Invest. **111**:1265–1273.

Raygada, J.L., and Levine, D.P.(2009). Methicillin-Resistant Staphylococcus aureus: A Growing Risk in the Hospital and in the Community. American Health & Drug Benefits **2**(2):86-95.

Niemz, M.H. (2007). Laser-Tissue Interactions: Fundamentals and Applications. 3rd Ed., Springer, Germany.

Gomer, C.J. (ed.) (2010). Photodynamic Therapy Methods and Protocols, Springer.USA.

Sharma, S.K., Chiang, L.Y. and Hamblin, M.R.(2011). Photodynamic therapy with fullerenes in vivo: reality or a dream?. Nanomedicine (Lond) **6**(10): 1813–1825.

Harley, J. P. and Prescott, L.M. (2002). Laboratory exercises in microbiology. 5th edition. McGraw Hill companies. New York.

Adams, M.R. and Moss M. O. (2000), „ Food Microbiology‟,2 nd ed. The Royal Society of Chemistry, Cambridge, UK. PP.447.

Vandepitte, J.; Verhaegen, J.; Engbaek, K.; Rohner, P.; Piot, P.; and Heuck, C.C. (2003). Basic Laboratory Procedures in Clinical Bacteriology. 2nd.ed. World Health Organization, Geneva.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Amer. J. Clin. Pathol. **45**:493-496.

Alwan, M. J., Lafta, I.J., Hamzah, A. M. (2011).Bacterial isolation from burn wound infections and studying their antimicrobial susceptibility. Kufa Journal For Veterinary Medical Sciences **2**:121-131.

Al-Taie, L. H., Hassan, S., Al-Mayah, K. Sh., Talib, S. (2014). Isolation and Identification of Bacterial Burn Wound Infection and Their Sensitivity to Antibiotics. Al- Mustansiriyah J. Sci. **25**: 17-24.

Qader, A.R. and Muhamad, J.A. (2010). Nosocomial Infection in Sulaimani Burn Hospital, Iraq. Ann Burns Fire Disasters. **23**: 177-181.

Al-Zubaidy, S.K. and Maki, A.M.(2015). The Effect of 532 nm Diode Pumped Solid State (DPSS) Laser in Combination with Safranin on the Growth of Pseudomonas aeruginosa and Staphylococcus aureus in Vitro. Iraqi J. Laser, Part B, **14**:11-18.

Dadras, S., Mohajerani, E., Eftekhar, F. and Hosseini, M.(2006). Different photoresponses of Staphylococcus aureus and Pseudomonas aeruginosa to 514, 532, and 633 nm low level lasers in vitro. Curr Microbiol **53**(4):282–6.

Kim, S., Kim, J., Lim, W., Jeon, S., Kim, O., Koh, J., Kim, C., Choi, H., and Kim, O.(2013). In Vitro Bactericidal Effects of 625, 525, and 425 nm Wavelength (ed, Green, and lue) Light-Emitting Diode Irradiation. Photomed Laser Surg. **31**(11): 554–562.

Kashef, N., Abadi, G. R. S. and Djavid, G. E. (2012). Phototoxicity of phenothiazinium dyes against methicillin-resistant Staphylococcus aureus and multi-drug resistant Escherichia coli. Photodiagnosis and Photodynamic Therapy.**9**: 11-15.

Ismael, D.K (2014).study of photodynamic effect of (632nm)laser diode light on *staphylococcus aureus* using Methylene blue as photosensitizer. M.Sc. thesis. Institute of

Genetic Engineering and Biotechnology for Post Graduate Studies, Baghdad University.

Silva, D.C., Plapler, H., Costa, M.M., Silva, S.R., Sá Mda, C. and Silva, B.S.(2013). Low level laser therapy (AlGaInP) applied at 5 J/cm2 reduces the proliferation of Staphylococcus aureus MRSA in infected wounds and intact skin in rats. An Bras Dermatol **88**(1):50-5.

التأثير الديناميكي الضوئي المضاد للبكتريا لليزر الحالة الصلبة المضخ بالدايود ذو الطول الموجي 532 نانومتر وليزر الدايود بطول موجي 650 نانومتر على نمو بكتريا المكورات العنقوديةالذهبية المقاومة للمثيسيلين المعزولة من اخماج الجروح والحروق

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الخلاصة: تم في هذة الدراسة بحث التأثيرالديناميكي الضوئي ضد بكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين باستخدام ليزرين مختلفين (MRSA)، ليزر الحالة الصلبة المضخ بالدايود DPSS بطول موجي 532 نانومتر بوجود المتحسس الضوئي سفرانين O وليزر الدايود بطول موجي 650 نانومتر بوجود المتحسس الضوئي ازرق المثيلين. جمعت مائة عينة مسحية من اخماج الحروق والجروح لمرضى راقدين في مستشفيين في بغداد (مستشفى الحروق التخصصي في مدينة الطب ومستشفى الامامين الجوادين التعليمي) خلال الفترة من كانون الاول 2015 الى شباط 2016 تم التحري عن حساسية البكتريا للمضادات الحيوية باستخدام طريقة كيربي بيور. تضمنت تجارب التشعيع اربعة مجاميع: مجموعة السيطرة، مجموعة المتحسس الضوئي فقط، مجموعة الليزر فقط ومجموعة الليزر بوجود المتحسس الضوئي. اظهرت النتائج بأن ليزر DPSS (532 نانومتر) وبكثافة قدرة 0.157 واط/سنتمتر مربع مع السفرانين O بتركيز 0.5 ملغم/مل كان اكثرتاثيرا في تقليل عدد خلايا MRSA من ليزر الدايود (650 نانومتر) وبكثافة قدرة 0.052 واط/ سنتمتر مربع بوجود ازرق الميثيلين بتركيز 0.1 ملغم/مل . تم قتل 100% من خلال MRSA بعد التعريض لليزر ال DPSS (532 نانومتر) بوجود المتحسس الضوئي سفرانين O خلال ثلاث دقائق فاكثر بينما استغرق 11 دقيقة للوصول الى نفس النتيجة عندما شععت بليزر الدايود (650 نانومتر) بوجود المتحسس الضوئي ازرق الميثيلين. وعليه يمكن اعتبار التاثير التثبيطي الديناميكي الضوئي كوسيلة بديلة لعلاج أخماج الجروح والحروق السطحية من خلال قتل الخلايا البكتيرية.