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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

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Abstract: The effect of 532nm Diode Pumped Solid State (DPSS) laser at power density of 5.234 W/cm^2 on the growth of Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* was evaluated. These bacteria were isolated from samples taken from burn and infected wound areas of 55 patients admitted to the burn-wound unit in Al-Kindy teaching hospital in Baghdad during the period from October 2012 to March 2013. Each isolate was identified using microscopic, cultural and biochemical methods. A standard bacterial suspension was prepared for each isolate. Serial dilutions were then prepared and a dilution of 10^{-5} was selected. Irradiation experiments included four groups: (L-P-) bacterial suspension in saline solution, (L-P+) bacterial suspension in the presence of 0.1mg/ml photosensitizer (safranin O), (L+P-) bacterial suspension treated with laser radiation only and finally (L+P+) bacterial suspension treated with laser radiation in the presence of the photosensitizer. After irradiation, Cetrimide agar (*P. aeruginosa)* and Mannitol salt agar (*S.aureus*) were used. Seven replicates were used for each experimental group. Different times of exposure were applied for irradiated groups. The results revealed that twenty two isolates out of 70 samples were positive for *P. aeruginosa* (31%) and fifteen isolates out of 35 samples were positive for *S. aureus* (42%). The combined effect of DPSS laser and safranine O was significantly effective in reducing the number of Colony Forming Units per milliliter (CFU/ml) of *P. aeruginosa* and *S. aureus* compared with control groups. Almost a complete bacterial mortality achieved at 25 and 5 minutes of exposing to laser light in the presence of safranin O for *P. aeruginosa* and *S. aureus* respectively, suggesting that singlet oxygen (O_2*) and/or reactive oxygen species (ROS) were involved in the killing of the bacteria.

Introduction

The worldwide increase in antibiotic resistance among different classes of gram-positive and gram-negative bacteria has led to search for alternative anti-microbial therapies, like anti-microbial Photodynamic Therapy (PDT) (Hamblin and Hasan, 2004; Maisch, 2007). At this time, there is no routine application of anti-microbial PDT in the treatment of localized infections in such areas as skin, wounds and periodontal pockets. However, if the resistance against antibiotics may become worst, anti-microbial PDT may be an alternative therapy option in clinical practice depending on the pharmacokinetics and the illumination time (Jaweetz *et al*., 2001; Maisch, 2007). Photodynamic therapy has been recognized as an alternative treatment for localized microbial infections, as it is effective against antibiotic-resistant microorganisms. The efficacy of antimicrobial PDT for different pathogens depends on the type and concentration of the photosensitizer (Donnelly *et al*., 2007)**.** The process requires the use of a chemical compound denominated the photo sensitizer (PS). The application of a light that corresponds

to the absorption band of PS and the presence of oxygen, promotes the formation of reactive radicals, such as singlet oxygen (Niemz, 2007)**.** PDT has previously been used to kill pathogenic microorganisms in vitro and that PS bearing a cationic charge or using such agents causes increasing permeability of the outer membrane will increase the efficacy of killing Gram negative bacteria. All the available evidence suggests that multi-antibiotic resistant strains are as easily killed by PDT, and that bacteria and fungi will not readily develop resistance to PDT (Hamblin and Hasan, 2004). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are among the most common pathogens isolated from both acute and chronic wounds and burns of various etiologies. These two species have proven particularly difficult to treat because they possess a large number of antimicrobial resistance genes and virulence factors (Church *et al.*, 2006; Murray & Hospenthal, 2008).

In this study we aim to evaluate the effect of diode pumped solid state (DPSS) (532 nm wavelength and 123 mW output power) laser irradiation on the growth of *P. aeruginosa* and *S. aureus* at different exposure times. This is to be compared with the effects of photosensitization by safranin O and the same laser irradiation.

Materials & Methods

Seventy samples were obtained from burn and infected wound areas using sterile disposable swabs in transport media. These samples were collected from 55 patients admitted to the burn-wound units in Al-Kindy teaching hospitals in Baghdad during the period from October 2012 to March 2013. *P.aeruginosa and S. aureus* isolates were identified on the basis of colony morphology, Gram staining, microscopic examination, biochemical tests (Greenwood *et al.*, 1997; Macfadden, 2000; Benson, 2002) and confirmed by Api 20 E and Api staph system respectively. One isolate of each species were selected from 27 isolates according to the pyocyanin production test for *P. aeruginosa* and to positive mannitol fermentation for *S.aureus*. The selected strains were grown aerobically on Cetrimide agar and Mannitol salt agar for *P. aeruginosa* and *S.aureus* respectively. A standard suspension of bacterial growth with dilution of 10^{-5} were prpared for each bacteria according to preliminary trials of viability count using spectrophotometer method. The experimental sample was prepared by vortex mixing 150µl of this suspension with equal volume of a 0.1 mg/ml filtered sterilized solution of safranin O in a sterile eppendorf tube. Sample was then subjected to laser irradiation experiment.

The laser used in this study was a Diode Pumped Solid State (DPSS) with 532 nm wavelength, 123 mW output powers and CW mode. The irradiation experiment included the following four groups all of which were performed in the dark:

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 0.1mg/ml photosensitizer only (safranin O). It was considered as a second control group.

Group III (L+P-): this was the one that 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.

After irradiation, an aliquot of 100 µl of the suspension was spread over the surface of Cetrimide agar plates (*P. aeruginosa)* and Mannitol salt agar plates (*S.aureus*) for each experimental group. Seven replicates were used for each assay. Plates were then incubated aerobically at 37 °C for 24 hrs.

Results

Bacterial isolates:

P.aeruginosa isolates gave pale colonies (as non-lactose fermentor) on MacConkey's agar. On Blood agar, the bacteria gave β-hemolysis. They grew and gave green colored colonies on King A agar at 37°C. All *P.aeruginosa* isolated colonies were semi-mucoid (Baron and Finegold, 1990; Macfaddin, 2000). While all *S.aureus* isolates on mannitol salt agar were yellow and may even turned the medium around the colony yellow due to the drop in pH around the colony of a mannitol fermenter. On Blood agar, the bacteria gave β-hemolysis. (Sritharan, 2006; Götz *et al*., 2006).

Laser irradiation:

The combined effect of 532nm DPSS laser and safranin O on the growth of *P. aeruginosa*

at different exposure times is shown in Figure 1 and Table 1. The results show that there is no significant differences in the number of log CFU/ml between the two control groups, L-P- (Neither laser nor photosensitizer) and L-P+ (phtosensitizer only). However, significant differences ($P<0.05$) in the number of log CFU/ml were detected between laser irradiated groups (L+P- and L+P+) and control groups (L-P- and L-P+). The number of log CFU/ml for L+P- group was significantly ($P<0.05$) lower (7.9) than the control groups (8.2) at 20 minutes exposure time, while the log CFU/ml of group L+P+ was significantly lower ($P<0.01$) than the control groups at all times of exposure. The lowest value of log CFU/ml was achieved at 25 minutes exposure time (2.4) (Table 1).

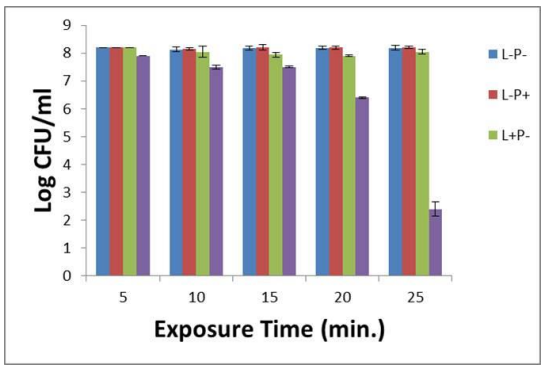

Fig. 1: Mean Log CFU/ml and standard deviation obtained for photosensitization of *P.aeruginosa* using 532 nm DPSS laser at power density of 5.234 W/cm2 and safranin O

Table 1: Mean, standard deviation and the Tukey's post hoc test of log CFU/ml obtained for photosensitized *P.aeruginosa* with safranin O using 532 nm DPSS laser at power density of 5.234 W/cm^2.

Time (min.)	Power density= 5.234 W/cm^2			
	L-P-	L-P+	L+P-	L+P+
5	8.20±0.09 a A	8.20±0.04 a A	8.20±0.2 a b A	7.90±0.07 b A
10	8.13±0.08 a A	8.15±0.1 a A	8.04±0.09 a A	7.49±0.03 b A
15	8.18±0.06 a A	8.20±0.06 a A	7.94±0.03 a A	7.50±0.03 b A
20	8.19±0.08 a A	8.20±0.05 a A	7.90±0.09 b A	6.40±0.26 c A
25	8.19±0.08 a A	8.20±0.05 a A	8.04±0.18 a A	2.40±3.3 b B

-Mean values followed by different small letters differed significantly ($P<0.05$) between experimental groups, L-P-,L-P+,L+P-and L+P+.
-Mean values followed by different capital letters differed significantly ($P<0.05$) between times, 5, 10 ,15 ,20 and 25.

The percentage of reduction of *P. aeruginosa* increased with increasing exposure time and reached the highest reduction percentage (99.8%) at 25 minutes exposure time in relation to the control group (L-P-) (Table 2).

Table 2: Percentage of reduction, deduced from mean values of CFU/ml for *P. aeruginosa* for groups submitted to laser irradiation in the presence of safranin O (L+P+) in relation to the groups with no exposure to laser light or photosensitizer (L-P-).

Time (min.)	Power density $^{=}$5.234 W/cm^2		
	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)
5	974X10 ^{5}	161,285,714	39.61
10	306 X10 ^{5}	135,714,286	77.45
15	298 X10 ^{5}	151,714,286	80.36
20	26 X10 ^{5}	156,428,571	98.34
25	4 X10 ^{5}	158,285,714	99.75

In the case *S. aureus*, Figure 2 and Table 3 show the effect of 532nm DPSS laser combined with the photosensitizer (safranin O) on the growth of *S. aureus* at different exposure times. Similar to the previous experiment on *P. aeruginosa*, The results showed that there is no significant difference in the number of log CFU/ml of between the two control groups (L-P-) and (L-P+) at all times of exposure (Fig.2 and Table 3). These results of both bacteria indicate that safranin O at concentration of 0.1 mg/ml had no lethal effect on the bacteria.

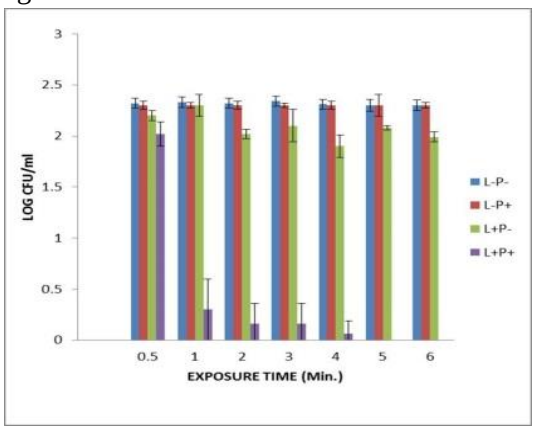

Fig. 2: Mean Log CFU/ml and standard deviation obtained for photosensitization of *S. aureus* using 532 nm DPSS laser at power density of 5.234 W/cm^2 and safranin O.

Significant differences (P<0.05) were observed in log CFU/ml of *S. aureus* between group L+P- (laser only) and the control groups (L-P- & L-P+) (Fig.2 and Table 3). These significant differences started at 2 minutes exposure time (2.02) compared with the control groups L-P- (2.32) and group L-P+ (2.30). The number of log CFU/ml was significantly decreased with increasing the time of exposure and reached a minimum (1.99) at 6 minutes exposure time (P<0.001) compared to the control groups (L-P- and L-P+) (2.3) (Fig.2 and Table 3).

Irradiation in the presence of safranin O (L+P+) conspicuously caused a significant decrease in the log CFU/ml of *S.aureus* compared to the control groups at all times of exposure (Fig.2 and Table 3). At 30 seconds exposure time, the number of log CFU/ml of group L+P+ (2.02) was significantly (P<0.05) lower than that for the groups, L-P- (2.32), L-P+ (2.30) and L+P- (2.20). The number of log CFU/ml for group L+P+ decreased with increasing exposure time and reached 0.06 at four minutes exposure time. This value is significantly (P<0.001) lower than that of groups L-P- (2.31), L-P+ (2.30) and L+P- (1.90).

Table 3: Mean, standard deviation and the Tukey's post hoc test of log CFU/ml obtained for *S. aureus*.

Time (min.)	Power density =5.234 W/cm^2			
	L-P-	**L-P+**	**L+P-**	**L+P+**
0.5	2.32±0.05 a A	2.30±0.04 a A	2.20±0.05 a BC	2.02±0.12 b A
1	2.33±0.05 a A	2.30±0.03 a A	2.30±0.11 a C	0.3±0.3 b B
2	2.32±0.05 a A	2.30±0.04 a A	2.02±0.045 b A	0.16±0.2 c B
3	2.34±0.05 a A	2.30±0.02 a A	2.10±0.16 b AB	0.16±0.2 c B
4	2.31±0.05 a A	2.30±0.04 a A	1.90±0.11 b A	0.06±0.13 c B
5	2.30±0.06 a A	2.30±0.11 a A	2.08±0.02 b B	0.00±0.00 c B
6	2.30±0.05 a A	2.30±0.03 a A	1.99±0.05 b A	0.00±0.00 c B

*Mean values followed by different small letters differed significantly (P<0.05) between experimental groups,L-P-,L-P+,L+P- and L+P+. **Mean values followed by different capital letters differed significantly (P<0.05) between times, 5, 10 ,15 ,20 and 25.

Table 4 shows the percentage of reduction in the bacterial number of CFU/ml for group L+P+ in relation to the group L-P-. The results revealed that the percentage of reduction in *S. aureus* growth increased with increasing the exposure time and reached 99.9% at 6 minutes exposure time compared to the control group L-P-.

Table 4: Percentage of reduction, deduced from mean values of CFU/ml for *S. aureus* for groups submitted to laser irradiation in the presence of safranin O (L+P+) in relation to the groups with no exposure to laser light or photosensitizer (L-P-).

Time (min.)	Power density =5.234 W/cm^2		
	Mean CFU/ml L+P+	**Mean CFU/ml L-P-**	**Reduction of CFU/ml (%)**
0.5	1102X10^4	21114286	47.81
1	22 X10^4	21842857	98.99
2	12 X10^4	21414286	99.44
3	12 X10^4	22028571	99.46
4	8 X10^4	20928571	99.62
5	4 X10^4	20385714	99.80
6	2 X10^4	21285714	99.91

Discussion

In this study, photoactivation of safranin O at concentration of 0.1 mg/ml, followed by 532 nm DPSS laser irradiation at power density of 5.234 W/cm^2 reduced the number of log CFU/ml of *P. aeruginosa* significantly in comparison with the control group. This result is in agreement with that of various studies (Dadras *et al.*, 2006; Street *et al.*, 2009; Thakuri *et al.*, 2011; Abbas *et al.*, 2013). Dadras *et al.* (2006) investigated the effect of SHG Nd:YAG (532nm) and 633 nm He-Ne lasers on the growth of *P. aeruginosa* and *S. aureus* in the presence of safranin O and Toluidine blue O. They observed a decrease in the population of *P.aeruginosa* compared to the non-sensitized irradiated and non-irradiated controls. Thakuri *et al.* (2011) studied the effect of blue light emitting diodes (LEDs) and Riboflavin as light source and photosensitizer for in vitro killing of *S. aureus* and *P. aeruginosa.*They found that combination of blue LEDs and Riboflavin significantly reduced ($P<0.05$) the number of both bacteria compared to all control samples that included: control untreated, control treated with light only and control treated with riboflavin only. Moreover, In the study by Street *et al.*(2009), the combination of 670 nm diode laser light and methylene blue caused 100% eradication of *P.aeruginosa* at >15.5 J/cm^2. Abbas (2013) studied the combined effect of frequency-doubled Nd:YAG laser radiation (532 nm) and Safranin O on the growth of *P. aeruginosa.* She found that the number of CFU/ml decreased with increasing the dose of irradiation. Complete killing of *P. aeruginosa* was observed at 1.831 J/cm^2 energy density for different times (5, 8 and 11 minutes).

In general, it was found that gram-negative bacteria are more resistant to photodynamic therapy than gram-positive bacteria (Maisch *et al.*, 2005). Gram-negative bacteria possess a tougher obstacle mainly due to their double-layer outer membrane structure (Baron, 1996). In our study, the bactericidal effect of 532 nm DPSS laser irradiation in the presence of safranin O was more effective on *S.aureus* than *P.aeruginosa*. This may be due to the structural differences of these two bacteria (Baron, 1996). *P.aeruginosa* has a thin cell wall surrounded by a semipermeable outer membrane, whereas *S.aureus* has a thick cell wall (Boyd, 1988).

The mechanism of cell destruction by laser has important implications in clinical therapy. Low-intensity laser light can cause acceleration of electron transfer in the respiratory chain of irradiated cell (Karu, 1988). At higher doses, this excitation energy is transferred to oxygen to form singlet oxygen. When cells are exposed to light without dye, the cytochromes and flavins of the electron transport chain serve as photosensitizers. The dyeing agents, which can absorb the radiation, bind to components of the cell and thereby enable more laser light to be absorbed (Karu, 1988). Reactive oxygen species (ROS) and singlet oxygen can be produced by type 1 and type 2 pathways of photosensitization reactions (St. Denis and Hamblin, 2011). Some researchers (Henderson and Dougherty, 1992; Tavares *et al.*, 2011) have reported that $^1O_2$ (Type II mechanism) plays a very important role in antimicrobial PDT. Other studies have shown that Type I mechanisms (particularly HO.) can also be equally important as Type II pathways (Nitzan *et al.*,1989; Ergaieg *et al.*, 2008). However these studies did not clearly discuss whether different reactive oxygen species (ROS) are more effective for killing Gram-positive or Gram-negative bacteria. More recently, Huang *et al.* (2012) suggested that Gram-negative bacteria are more susceptible to HO. while Gram-positive bacteria are more susceptible to $^1O_2$.

Conclusion

The results of this work suggest that 532 nm diode pump solid state laser (DPSS) radiation at 5.234 W/cm² power density significantly reduce bacterial number of Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* with clear higher rates regarding the former. Furthermore, the combination of DPSS laser radiation and safranin O was superior in the destruction of both bacterial cells (*S.aureus* and *P.aeruginosa*) compared with laser irradiation alone. Gram-positive *S.aureus* was more sensitive to photosensitization by DPSS laser irradiation with Safranin O than Gram-negative *P. aeruginosa.* This suggests that photo inactivation of Gram-negative and Gram-positive bacteria, in general, by photosensitization could be considered as an alternative approach to current antibacterial methods of treating burn and wound infections.

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التأثيرالمشترك لليزر الحالة الصلبة المضخ بالدايود (DPSS) 532 نانومتر مع سفرانين O على نمو *Pseudomonas aeruginosa* and *Staphylococcus aureus* خارج الجسم الحي

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الخلاصة: تم تقييم تأثير ليزر الحالة الصلبة المضخ بالدايود (DPSS) ذو الطول الموجي 532 نانوميتر عند كثافة طاقة 5.234 واط /سم^2 على نمو بكتريا الزوائف الزنجارية السالبة لصبغة غرام *Pseudomonas aeruginosa* وعلى المكورات العنقودية الذهبية الموجبة لصبغة غرام *Staphylococcus aureus*. عزلت هذه البكتريا من الحروق والجروح الملوثة ل 55 مريض تم ادخالهم الى وحدات الحروق والجروح في مستشفى الكندي التعليمي في بغداد اثناء الفترة من تشرين الاول 2012 الى آذار 2013. شخصت كل سلالة باستخدام الطرائق المعتمدة على الفحوصات المجهرية والصفات المظهرية و الكيموحيوية. حضر معلق بكتيري قياسي لكل سلالة. ثم حضرت تخافيف متسلسلة وأختير التخفيف 10^{-5} . تضمنت تجارب التشعيع اربعة مجاميع: (L-P-) معلق بكتيري في محلول ملحي, (L-P+) معلق بكتيري بوجود 0.1 ملغ /مل متحسس ضوئي (سفرانين O), (L+P-) معلق بكتيري معالج باشعاع الليزر فقط وأخيرا (L+P+) معلق بكتيري معالج باشعاع الليزر بوجود المتحسس الضوئي. بعد التشعيع استخدم وسطي السترمايد اكار Cetrimide agar لتنمية بكتريا *P. aeruginosa* و ملح المانيتول Mannitol salt agar لتنمية بكتريا *S. Aureus*. استخدمت سبع مكررات لكل مجموعة تجريبية مع تطبيق اوقات تشعيع مختلفة للمجاميع المشععة. اظهرت النتائج ان اثنان وعشرون عزلة من اصل 70 عينة كانت *P. aeruginosa* (31%) وخمسة عشر عزلة من اصل 35 عينة كانت *S. aureus* (42%). كان التأثير المشترك لليزر الحالة الصلبة المضخ بالدايود و سفرانين O فعال معنويا في خفض عدد وحدات تكوين المستعمرة في الملتر (CFU/ml) ل *P. aeruginosa* و *S. aureus* مقارنة مع مجموعات السيطرة. حققت وفيات بكتريا كاملة تقريبا في الدقيقة 25 و 5 من التعريض لضوء الليزر في وجود السفرانين O ل *P. aeruginosa* و *S. aureus* على التوالي مما يدل على ان الاوكسجين المفرد (O_2*) مع/او انواع الاوكسجين الفعالة (ROS) لها علاقة بقتل البكتريا .