Iraqi J. Laser, Part B, Vol.12, pp. 33-40 (2013)

IRAQI JOURNAL OF LASER

The Combined Effect of Q-Switched Nd:YAG Laser (532 Nm) and Safranin O on the Growth of *Pseudomonas Aeruginosa*and its Susceptibilityto Antibiotics and Pyocyanin Production

Hanan M. Abbas **Amel M. Maki** and **Nuha Kandala**

Institute of Laser for Postgraduate Studies, University of Baghdad, Baghdad, Iraq

(Received 5 May 2013; accepted 30 June 2013)

Abstract: Twenty five samples out of sixty wound swabs taken from burn patients were identified as *P. aeruginosa*bacteria by conventional methods. Antibiotics susceptibility tests were performed against thirteen antibiotics. *P. aeruginosa* samples were treated with 0.5 mg/ml of Safranin O solution then irradiated with 532nm Q-switched Nd:YAG laser at four energy densities (0.324, 0.704, 1.380, and 1.831 J/cm^2) for different times of 5, 8 and 11 minutes with 5Hz repetition rate. The viability, susceptibility to antibiotic and production of pyocyanin were determined before and after irradiation. The results showed that the number of CFU/ml of *P. aeruginosa* decreased with increasing the dose of irradiation. Complete killing of cells was observed at 1.831 J/cm^2 energy density for different times (5, 8 and 11 minutes). The susceptibility of *P. aeruginosa*to antibiotics increased with increasing the dose of irradiation, especially to Oxacillin and Azithromycin antibiotics, except in the case of Ceftazidime, where no variation in the susceptibility at any dose was observed. No change was observed in the production of pyocyanin.

Introduction

*Pseudomonas aeruginosa*is one of the most commonly considered pathogensthat cause fatal wound infections. Most strains of *P. aeruginosa* have multiple virulence factors that include exotoxins and enzyme (Sadikot*et al.*, 2005). During the 1960s, *P. aeruginosa* emerged as a major human pathogen (Mandell*et al.*, 2009). Since then, this pathogen has become the gram-negative bacteria most commonly associated with hospital-acquired infections (Micek *et al.*, 2005) with a mortality rate of 18–61%.

These infections require optimal management, which remains the subject of considerable controversy (Kang *et al*, 2003).Further, the reason for complicating the management of patients is the limited number of drugs available for treatment of *P. aeruginosa* infections because of the inherent resistance of these bacteria to multiple antibiotics. The ability of *P. aeruginosa* to use numerous mechanisms of antibacterial resistance and its ability to acquire additional determinants further compound the complexity of managing these patients (Livermore, 2002; Hancock, 1998). The presence of pyocyanin, a *Pseudomonas* secreted toxin, is easy to detect due to its blue-green color that turns stationary phase cultures of *P. aeruginosa* green, and is commonly found to stain infected tissues, pus, or dressings (Cox, 1986). Recently, Muller et al. (2009) examined wound dressings from burn patients infected with *P. aeruginosa* and found that four of seven dressings contained pyocyanin. New therapeutic approaches are urgently needed to combat such multiple antibiotic resistant bacteria. Bactericidal effect of different lasers on gram positive and gram negative bacteria were demonstrated by distinct authors' worldwide (Bertoloni *et al*., 1993; DeSimon *et al.*, 1999).The thermal effect of green light from

second harmonic generation (SHG) Nd:YAG laser on *P. aeruginosa* were monitored. Previous studies had shown the effectiveness of Q-switched Nd:YAG laser as bactericidal too (Ward et al.,1996; Meral*et al*., 2003). Because infection is a common cause of delayed wound healing, it is important to understand the effect of laser irradiation on bacterial growth.Factors like energy density, time, light wavelength, beam diameter, population and type of bacteria in the irradiation suspension affect minimum bactericidal energy level and indicate whether the resulting effect is due to photochemical mechanism or photothermal mechanism(Andres *et al*., 2000). However, the literature includes few laser studies in that regard and more systematic investigations are needed towards an optimum and effective combination of dose parameters.The objective of present project was to investigate the combined effect of photosensitizer (Safranin O) and Frequency-doubled Nd:YAG laser irradiation at 532 nm on the growth of *P. aeruginosa* at different number of pulses and different energy densities and to evaluate the activity of *P. aeruginosa* isolate to produce pyocyanin and its susceptibility to antibiotics.

Materials and Method

Bacterial Specimens

Swap samples were taken from burn and wound areas (using sterile disposable swabs in transport media) of patients whose wound infection with *Pseudomonas*is suspected. These samples were collected from patients hospitalized at Al-Kindi and Al-Wassity teaching hospitals in Baghdad during the period from January 2012 to February 2012.

P. aeruginosa isolates were isolated and identified using microscopic (Colle*et al*., 1996; Macfaddin, 2000), cultural, and biochemical methods (Cruikshank, 1976; Baron and Finegold, 1990; Collee *et al*., 1996; Macfaddin, 2000; Atlas and Synder, 2006) in addition toApi 20 E test.

Antibiotic Susceptibility Test

Thirteen various available and commonly used antibiotics disks were used in this test. The susceptibility of 25 isolates of *P. aeruginosa*to different antimicrobials was determined by Kirby-Bauer disk diffusion methods on Muller-Hinton agar (Bauer et al 1966). The sensitivity and resistance were determined by measuring the diameter of inhibition zones around the antibiotic disc according to National Committee for Clinical Laboratory Standards (NCCLs, 2006). The antibiotics discsthat wereused included: Amikacin (10 µg), Azithromycin (15 µg), Cefotaxime (10 µg), Gentamicin (30 µg), Imipenem (10 µg), Oxacillin (10 µg), Penicillin (10 µg), Tetracycline (10 µg) and Tobramycin (10 µg) from Bio analyze, Turkey and Cefepime (50 µg), Ceftazidime(30 µg), Ceftriaxone (30 µg) and Meropenem (10 µg) from Himedia, India.

Bacterial Laser Irradiation: Viability Test

One isolate of *P. aeruginosa* was selected according to the resistance test to several antibiotics and the pyocyanin production test. Standard suspensions of bacterial growth with dilution of 10^{-6} were prepared. An amount of 150 ml of this suspension was transferred to a sterile appendrof tube that containing equal volume of a filtered sterilized solution of Safranin O at 0.5 mg/ml and mixed together by vortex. Sample was then subjected to laser irradiation experiment. The laser system used in the experiment was 532 nm Frequency-doubled Q-switched Nd:YAG laser.The irradiation experiment included the following four groups all of which were performed in the dark:

Group 1 (L-P-): this group was considered the control group. It was subjected to neither laser nor Photosensitizer.

Group 2 (L-P+): this group was treated with 0.5 mg/ml Photosensitizer only.

Group 3 (L+P-): this group was treated with laser radiation only without adding the Photosensitizer.

Group 4(L+P+): this group was irradiated with laser light in the presence of the Photosensitizer.

Irradiated groups were subjected to four fluences, 0.324, 0.704, 1.380 and1.831 J/cm² at 5, 8, and 11 minutes with a constant repetition rate of 5 Hz. After the irradiation, an aliquot of 100 µl of the suspension was spread over the surface of Cetrimide agar plates for each experimental group. Plates were then incubated aerobically at 37 °C for 24 hrs. Three replicates were used for each assay.

Bacterial Laser Irradiation: Antibiotic sensitivity Test

According to the results of irradiation experiment for viability test, afluence of 0.704 J/cm² was applied at 5, 8 and 11 minutes with a constant repetition rate of 5 Hz.

For antibiotic sensitivity test, a high concentrated suspension is needed to perform this test.Accordingly the standard suspension of 10^8cell/ml was used instead of 10^{-6}dilution. An aliquot of 150 µl of this suspension was mixed with 150 µl of safranin O in a sterile appendrof tube.

This suspension was then irradiated with laser at the above-mentioned fluence and time. After irradiation, 100 µl of the irradiated suspension was spread on Muller-Hintone agar plate in three different directions. Thirteen antibiotic disks were placed on the surface of the agar plates (6 – 7 disks for each plate). The plates were then incubated at 37°C for 24 hrs. After the incubation, the zone of inhibition was measured in for each disk. The results were compared with that of the control group (suspension without irradiation).

Bacterial Laser Irradiation:Pyocyanin Production Test

The bacterial preparation and laser irradiation parameters were similar to the previous section. The modifications on this procedure include firstly, taking 40 µl of the irradiated or non-irradiated standard bacterial suspensions with sterile micropipette and secondly, spotting this amount on the plate containing citrimide agar. Two spots were performed for each plate, one for irradiated and the other for control suspension. Three replicates were done and incubated at 37°C for 24 hrs. After incubation, change in color was observed.

The number of colony forming units per milliliter (CFU/ml) was analyzed using the SPSS statistical software package and Microsoft Office Excel. 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

Bacterial isolates

The results of bacterial identification tests of *P. aeruginosa* isolates gave mucoidpale colonies (as non-lactose fermentor) on MacConkey's agar, β-hemolysis on Blood agar. They grew and gave green colored colonies on King A agar at 37°C. They weregram negative bacteria, non-capsular, non-spore-forming, and positive foroxidase, catalase, and hemolysin tests. Also the results were positive for methyl red, citrate utilization, gelatin liquefaction, urease tests and Protease test. Negative results appeared for Indol, Voges-Proskaure, andKligler test. The results of Api20Etestconfirmed the biochemical identification of *P. aeruginosa*.All these results revealed that 25 isolates out of 60 samples being *P. aeruginosa*(41%).

Antibiotic Susceptibility of P. aeruginosa

The data obtained showed that all *P. aeruginosa*isolates were completely sensitive to Meropenen, Imipenem and Gentamicin. Therefore these three antibiotics can be considered as the most effective drug used in the present study.By contrast, the highest resistantrate of *P. aeruginosa*isolates was found against penicillin(96%),Oxacillin (96%), Tetracycline (92%) andCefotaxime (92%).For the remaining antibiotics, Ceftriaxone and Amikacin (36%), Tobramycin, Ceftazidime andCefipime(16%) and Azithromycin (12%) the isolates showed less resistance.

Laser Irradiation Result

For viability test, Fig.1 shows that mean values of log CFU/ml of *P. aeruginosa* for the four experimental groups(L+P+, L+P-, L-P+ and L-P-) at different times 5, 8, and 11 min for 0.324 J/cm^2 energy density. In the case where both the laser irradiation and the photosensitizer were involved in the interaction (group L+P+), the results indicated that the log CFU/ml decreased significantly (P<0.01) compared to that of the other three groups for each time (Fig. 1).The lowest log CFU/ml was recorded to be 8.41 at 11 min. The % of CFU/ml reduction was 85% (Table 1).

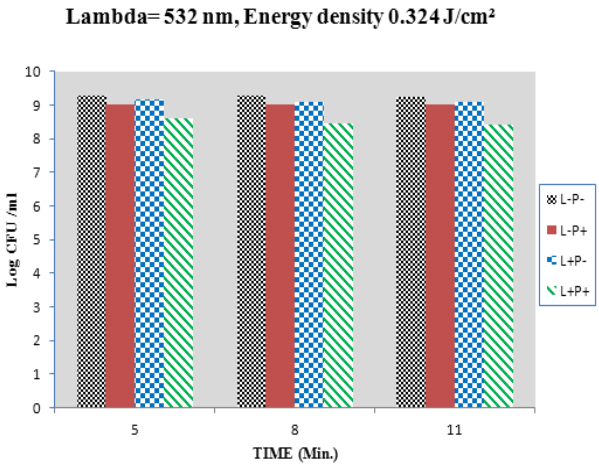

Fig. 1: Mean log CFU/ml vs. time at 0.324 J/cm2 energy density of P.

Table 1: Percentage of reduction for *P. aeruginosa*, deduced from mean values of CFU/ml after irradiation by frequency-doubled Nd:YAG laser.

Time (min.)	Energy density = 0.324 J/cm^2			Energy density =0.704 J/cm^2		
	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)
5	$4*10^8$	$19.433*10^8$	79.42	$5.5333*10^8$	$10.8**10^8$	48.77
8	$3.1667*10^8$	$18.5*10^8$	82.88	$5.3333*10^8$	$18.5*10^8$	71.17
11	$2.6667*10^8$	$18.033*10^8$	85.21	$1.2333*10^8$	$7.3*10^8$	83.11
Time (min.)	**Energy density = 1.380 J/cm^2**			**Energy density = 1.831 J/cm^2**		
	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)	Mean CFU/ml L+P+	Mean CFU/ml L-P-	Reduction of CFU/ml (%)
5	$1.6333*10^8$	$7.3*10^8$	77.63	.000	$12.967*10^8$	100
8	$2.3333*10^8$	$15*10^8$	98.44	.000	$12.967*10^8$	100
11	$3.3333*10^8$	$15*10^8$	99.78	.000	$10.8*10^8$	100

Figure 2 shows the mean values of log CFU/ml of *P. aeruginosa* for the four experimental groups L-P-, L-P+, L+P-, and L+P+ at different times 5, 8, and 11 min under 0.704J/cm^2 energy density.

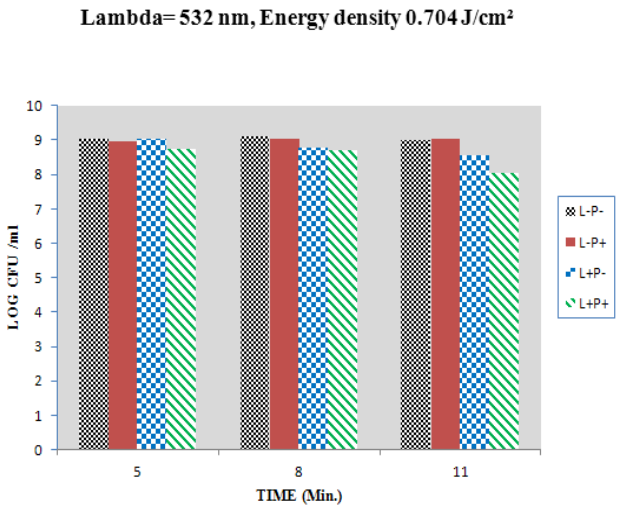

Fig. (2): Mean log CFU/ml vs. time at 0.704 J/cm2 energy density of P.

Significant reduction of log CFU/ml in relation to time was detected. At time 11 minutes the two groups L+P- (8.56) and L+P+ (8.05) were significantly (P<0.001) less compare to time 5 and 8. Furthermore, group L+P+ had the highest percentage of CFU/ml reduction (83.11%) at time 11 minutes in relation group L-P- in comparison to time 5 minutes (48.77%) and 8 minutes (71.17%) (Table1). The mean values of log CFU/ml of *P. aeruginosa* for four experimental groups L-P-, L-P+, L+P-, and L+P+ at different times 5, 8, and 11 min under 1.380J/cm^2 energy density are shown in Fig. 3. Group L+P+ showed a remarkable significant difference (P<0.0001) in log CFU/ml between times employed in this study. Log CFU/ml at time 11 (2.33) was significantly less than that at time 8minutes (5.03), which in turn was less than that at time 5 minutes (8.21).

At the same energy density of 1.380 J/cm^2, group L+P+ had significantly (P<0.0001) the lowest log CFU/ml compared to that of the other three experimental groups at times 8 and 11 minutes. The percentage of CFU reduction of

group (L+P+) reached 77.63%, 98.44% and 99.78% for 5, 8 and 11 minutes time respectively in comparison to the other groups (Table 1).

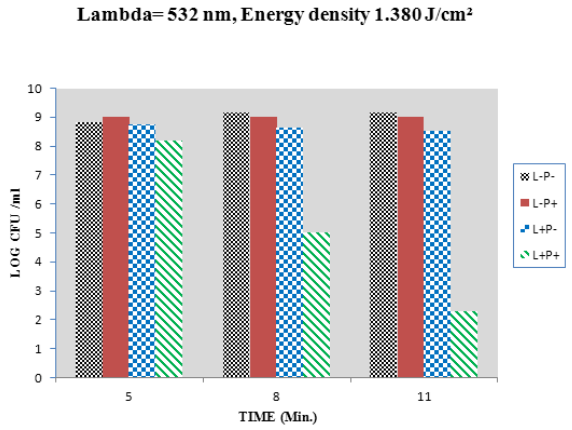

Fig. (3): Mean log CFU/ml vs. time at 1.380 J/cm2 energy density of P.

On the other hand, Fig. 4 shows the mean values of log CFU/ml of *P. aeruginosa* for the four experimental groups L-P-, L-P+, L+P-, and L+P+ at different times 5, 8, and 11 min under1.831 J/cm^2 energy density. In this case, all the experimental groups L-P-, L-P+, L+P- and L+P+ showed no significant differences in log CFU/ml between times for each group. Considering the differences between groups at each time, group L+P- showed significant ($P<0.05$) decrease in log CFU/ml in comparison with the other two groups, L-P- and L-P+, at all times. On the other hand, group L+P+ showed an exceptional significant ($P<0.0001$) reduction in log CFU/ml. In other words, the percentage of CFU/ml reduction had reached (100%) for times 5, 8, and 11 min, this means that 100% killing of the cells was achieved for group L+P+ at all time (Table1).

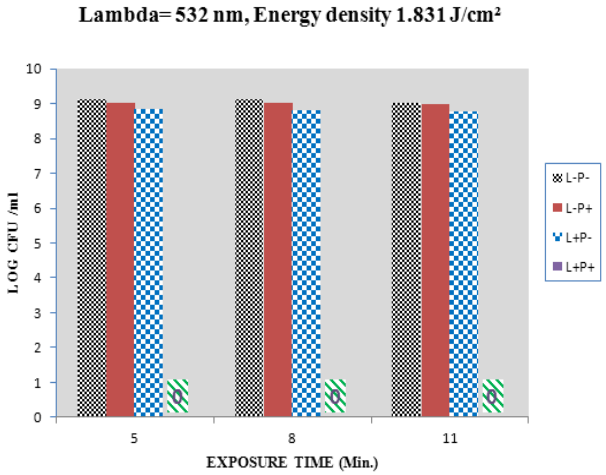

Fig. 4: Mean log CFU/ml vs. time at 1.831 J/cm2energy density of P.

Results of antibiotic sensitivity test after irradiation of *P. aeruginosa*isolate with energy density equal 0.636 at 180sec time showed slight changes in sensitivity to antibiotics .These changes increased mostly with time,the resistant isolates to some antibiotics became sensitive or intermediate. Ceftazidime, which showed no changes in the susceptibility with increasing time as shown in Table 2, which was an exception compared with the others. The results of the detection of pyocyanin production after the irradiation showed that there is no significant change between the irradiated and the control (without irradiation) isolate of *P. aeruginosa*.

Table (2): Susceptibility of *P. aeruginosa*to antibiotics after irradiation by frequency-doubled Nd:YAG laser .

Antibiotics	Zone of Inhibition (mm)			
	Control	Irradiation for 5 min	Irradiation for 8 min	Irradiation for 11 min
AK	16 (In)	18 (S)	18 (S)	20 (S)
CTX	12 (R)	12 (R)	13 (R)	14 (R)
OX	0 (R)	7 (R)	13 (S)	14 (S)
GN	25 (S)	25 (S)	25 (S)	24 (S)
IPM	17 (S)	20 (S)	20 (S)	20 (S)
TE	0 (R)	8 (R)	12 (R)	13 (R)

P	12 (R)	13 (R)	15 (R)	16 (R)
AZM	13 (R)	13 (R)	16 (In)	17 (In)
TOB	15 (S)	16 (S)	21 (S)	16 (S)
CTR	22 (S)	24 (S)	26 (S)	28 (S)
MRP	32 (S)	39 (S)	41 (S)	39 (S)
CAZ	0 (R)	8 (R)	1 (R)	6 (R)
CPM	0 (R)	0 (R)	0 (R)	0 (R)

Discussion

The reduction in CFU/ml, for groups irradiated with the laser only without photosensitizer, could be due to the presence of natural photo acceptors such as flavoproteins. It was found that flavoproteins are photo acceptors in visible light (Vo-Dinh, 2003) and after the light absorption, flavoproteins are converted into photosensitizers.

It is noticed from the present results that the mortality reached 100% for group L+P+ when the energy density increased to 1.831 J/cm^2.Photodynamic therapy is associated with presence of photosensitizer. Photodynamic therapy may arise from Photothermal interaction mechanism or photochemical interaction mechanism between light and tissues or cells. The latter, is characterized by the requirement of oxygen to interact with the target cells or tissues. On the other hand, in photothermal photodynamic therapy the photosensitizer is excited by the light and consequently the sensitizer is lifted to an excited state where it then releases vibrational energy (heat). The heat is the actual method of therapy that kills the targeted cells. We strongly believe that in our case the effect of the laser light and the resulting outcomes are due to photothermal interaction mechanism. Recently, *P. aeruginosa* has become increasingly resistant to various antimicrobial agents (Hocquet*et al.*, 2007; Bassetti*et al.*, 2008). Compared with other pathogens, *P. aeruginosa* is very difficult to eradicate as it displays high intrinsic resistance to a wide variety of antibiotics, including aminoglycosides, fluoroquinolones and B-lactams (Imipenem and Ceftazidime) (Gaynes and Edwards, 2005). The increase in the bacterial resistance to antibiotics makes the development of alternative antibacterial techniques an important area of research (Malik *et al.*, 1990).Some studies suggest that laser-induced effects in case of laser continuous irradiation is different from that of the case of pulsed laser irradiation (Karu, 1998; van Breugel and Dop Bar, 1992). Another possible explanation for these results may be attributed to the change in bacterial pumping system (efflux pump) that mainly responsible for bacterial resistance to antibiotics such as (β-Lactams, aminoglycoside) (livermore, 2002). A decrease in growth of bacteria and increase sensitivity of *P. aeruginosa*to antibiotics after irradiation may be an additional benefit of using light in the treatment of infectious disease.

No irradiation effect on pyocyanin production by *P. aeruginosa*was found in the current study. A negative effect of 805 nm diode laser irradiation on the ability of *P. aeruginosa* to produce pyocyaninwas also observed by Al-Rassam (2010).

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التأثير المشترك لليزر النيديميوم ياك ذو المفتاحية مضاعف التردد 532 نانومتر وسفرانين O على نمو *P.aeruginosa* وحساسيتها للمضادات الحيوية وانتاج البايوسين

حنان عباس امل مصطفى مكي نهى قندلا

معهد الليزر للدراسات العليا، جامعة بغداد، بغداد ، العراق

الخلاصة: تم تشخيص 25 عينة من 60 مسحة للجروح مأخوذة من مرضى مصابين بالحروق كبكتريا *P.aeruginosa* بالطرق التقليدية. تم اجريت اختبارات الحساسية للمضادات الحيوية لثلاثين مضاد حيوي. عوملت *P.aeruginosa* ب 0.5 ملغم/مل من محلول سفرانين O ثم شععت بليزر النيديميوم ياك ذو المفتاحية مضاعف التردد 532 نانومتر بأربعة كثافات طاقة (0.324, 0.704, 1.380 و 1.831 جول/سم^2) بثلاثة ازمان تعريضية 5, 8, و 11 دقيقة بمعدل تكرار 5 هيرتز. حددت الحيوية والحساسية للمضادات الحيوية وانتاج البايوسيانين قبل وبعد التشعيع. لوحظ قتل كلي للخلايا عند كثافة طاقة 1.831 جول/سم^2 بأزمان مختلفة (5, 8 و 11 دقيقة). ازدادت حساسية *P.aeruginosa* للمضادات الحيوية مع زيادة جرعة التشعيع وبالأخص للمضادات أوكساسلين و أزيثرومايسين ,ما عدا في حالة سيفلازيدايم, حيث لم يلاحظ أي تغير في الحساسية لأي جرعة. لم يلاحظ أي تغير في انتاج البايوسيانين.