Video Article

The Synergistic Effect of Visible Light and Gentamycin on *Pseudomona aeruginosa* Microorganisms

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Abstract

Recently there were several publications on the bactericidal effect of visible light, most of them claiming that blue part of the spectrum (400 nm-500 nm) is responsible for killing various pathogens[1-5]. The phototoxic effect of blue light was suggested to be a result of light-induced reactive oxygen species (ROS) formation by endogenous bacterial photosensitizers which mostly absorb light in the blue region[4,6,7]. There are also reports of biocidal effect of red and near infra red[8] as well as green light[9].

In the present study, we developed a method that allowed us to characterize the effect of high power green (wavelength of 532 nm) continuous (CW) and pulsed Q-switched (Q-S) light on *Pseudomonas aeruginosa*. Using this method we also studied the effect of green light combined with antibiotic treatment (gentamycin) on the bacteria viability. *P. aeruginosa* is a common noscomial opportunistic pathogen causing various diseases. The strain is fairly resistant to various antibiotics and contains many predicted AcrB/Mex-type RND multidrug efflux systems[10].

The method utilized free-living stationary phase Gram-negative bacteria (*P. aeruginosa* strain PAO1), grown in Luria Broth (LB) medium exposed to Q-switched and/or CW lasers with and without the addition of the antibiotic gentamycin. Cell viability was determined at different time points. The obtained results showed that laser treatment alone did not reduce cell viability compared to untreated control and that gentamycin treatment alone only resulted in a 0.5 log reduction in the viable count for *P. aeruginosa*. The combined laser and gentamycin treatment, however, resulted in a synergistic effect and the viability of *P. aeruginosa* was reduced by 8 log's.

The proposed method can further be implemented via the development of catheter like device capable of injecting an antibiotic solution into the infected organ while simultaneously illuminating the area with light.

Video Link

The video component of this article can be found at http://www.jove.com/video/4370/

Protocol

1. Bacterial Culture

1. Gram-negative *P. aeruginosa* strain PAO1 were grown in Luria Broth (LB) at 37 °C for 18 hr.
2. The culture of cells was then centrifuged at 7,500 rpm (rounds per minute) for 5 min and supernatant was removed.
3. The bacteria were resuspended in 10% LB and re-grown for another 2 hr to allow the culture to reenter stationary phase.
4. The bacteria suspension was then divided into two groups: in the first group (2 tubes) no antibiotic were added, in the second group we added the gentamycin antibiotic (50 µg/ml).

2. Determination of Colony-forming Units (CFU)

1. To determine cell viability 20 µl samples were taken from the experiment approximately every 2 hr within the time frame of 24 hr. Serial dilution of the samples were made and plated on LB agar plates and incubated overnight at 37 °C.
2. For each treatment, the CFUs per plate was determined and a comparison was made between the time periods and various treatments. The log reduction in CFU was calculated as described in Eq. (1):

Log reduction= LogU-LogC[CFU/ml]

Where U is the colony forming units value at each time point; CFU is the colony-forming unit while the units of CFU/ml equal to:

CFU/ml = (number of colonies x dilution factor)/(volume inoculated)

And C is the CFU found in the control sample at start time. Note that U designates the colony forming factor at measurement instant.

3. The dilution factor is the number of dilutions while in each one of them the concentration of the bacteria was reduced by a factor of 10. The inoculated volume was always 200 micro liters and it is related to the size of our test tube.

Therefore to summarize the concentration point of view, the gentamycin antibiotic was at concentration of 50 µg/ml. Regarding the bacteria, until the end of the process we had overall 8 dilutions. Each dilution was by a factor of 10 and it was done in tubes of 200 µl. The starting point was 20 µl of samples added into the 200 µl tube (and thus the initial concentration was $20/200C_0$=0.1C0 with C_0 being the initial concentration in the 20 µl of samples) and the final concentration was reduced by 8 orders of magnitude due to the 8 dilutions.

3. Illumination

1. CW Nd:YAG laser (wavelength of 532 nm and average optical power of 200 mW) was split into two optical paths using optical 50%/50% beam splitter. The beam diameter was about 10 mm. The exposure duration was 24 hr.
2. Q-switched pulsed Nd:YAG laser (wavelength of 532 nm, average power of 300 mW and optical peak power of 2.5 MW) was also split into two paths using optical 50%/50% beam splitter. The spot diameter was 6 mm. The pulse width of the Q-switched laser was 6 nsec and the repetition rate was 15Hz. The average power density was 106 mW/cm^2 and the peak power density was 8.83 kW/mm^2. The exposure duration was 24 hr.

Note that the bacterial suspension stirred during irradiation and it was kept under appropriate culture conditions for bacterial growth (in all tubes there was Luria Broth medium to allow the bacteria to grow).

Representative Results

The laser based setup is schematically presented in **Figure 1**. The first experimental condition utilized a CW Nd:YAG laser having wavelength of 532 nm (the second harmonic of the Nd:YAG) and average optical power of 200 mW. This beam was split into two optical paths using optical 50%/50% beam splitter such that each split beam had power of 100 mW. The beam diameter was about 10 mm and thus the power density was about 100 mW/cm^2. The exposure duration was 24 hr. Although the illumination power is relatively high it is not high enough to cause heating of the sample.

The second experimental condition utilized a Q-switched pulsed Nd:YAG laser having wavelength of 532 nm (second harmonic) and spot diameter of 6 mm. The average power was 300 mW and optical peak power of 2.5 MW. The pulse width of the Q-switched laser was 6 nsec and the repetition rate was 15Hz. This beam was also split into two paths using optical 50%/50% beam splitter. The average power density was about 100 mW/cm^2 and the peak power density was 8.83 kW/mm^2. This peak power density is equivalent to energy fluency of 88.3 µJ/mm^2 per pulse as each pulse was 10 nsec long in the time domain. The exposure duration was 24 hr. Both experiments were carried out under similar growth conditions with no-light exposure (*i.e.* with and without gentamycin) which served as a control.

In **Figures 2(a) and 2(b)** the effect obtained due to illumination of the samples with CW laser light and with Q-switched laser respectively with and without antibiotic on *P. aeruginosa* is presented.

In the samples that were not exposed to light (*i.e.* the control) there was no reduction in cell viability with or without gentamycin treatment. This result suggests that the bacteria are resistant to the gentamycin treatment, mimicking the situation often encountered in the clinic.

The laser light alone also did not induce any killing either. However, the combination of laser light and gentamycin reduced bacteria viability by several orders of magnitude. The most prominent effect was measured after 24 hr in which the combination of either CW or Q-switched laser reduced the viability by 8 orders of magnitude compared to the measurements obtained in the control group (antibiotic alone or light alone).

This is an important result that may suggest a solution of treatment for this type of antibiotic resistant bacteria. The fact that several hours are required in order to obtain effective killing of the bacteria does not reduce the clinical potential of this approach since the proposed treatment can be incorporated into catheters and other devices used in hospital. For instance in **Figure 3** we present an example of a designed catheter in which in addition to the liquid injection channel there are plurality of holes allowing to simultaneously diffuse proper illumination into the infected organ.

The number of samples used for the statistics was 6 (there was one or two cases of some of the tubes that were accidently contaminated and then they were taken out of the statistics). The *p* value was below 0.05.

Note that we did not repeat our experiments for different concentration levels of the antibiotics. In all of our experiments the concentration was very high. The reason for that was to better demonstrate the strength of our approach as if in the highest concentration the bacteria was still not affected from the antibiotics without illumination and was destroyed with the illumination, it will obviously happen for lower concentrations.

One of the rationales for choosing the illumination wavelength was to choose a wavelength for which the bacteria and the antibiotics are transparent. This is demonstrated in **Figure 4**. Additional motivation for using the 532 nm wavelength laser was due to its availability in our lab

and due to the fact that it allowed us also to obtain higher illumination power (compared to regular white light source with spectral filters) as well as tuning capability for the power and for the temporal behavior of the illumination.

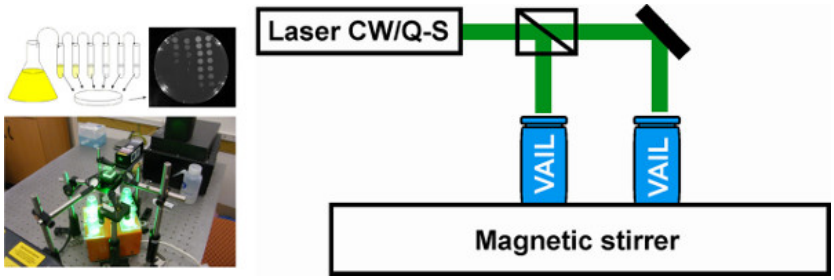

Figure 1. Bacteria illumination setup. The laser was either CW Nd:YAG laser or Q-switched pulsed Nd:YAG laser. On the left one may see the image of the experimental setup in which the laser is split between two tubes, one with antibiotics and one without it, in order to illuminate both of them in identical conditions. Both tubes are positioned on a stirrer. Schematic sketch of the experimental setup is seen in the right part of the figure.

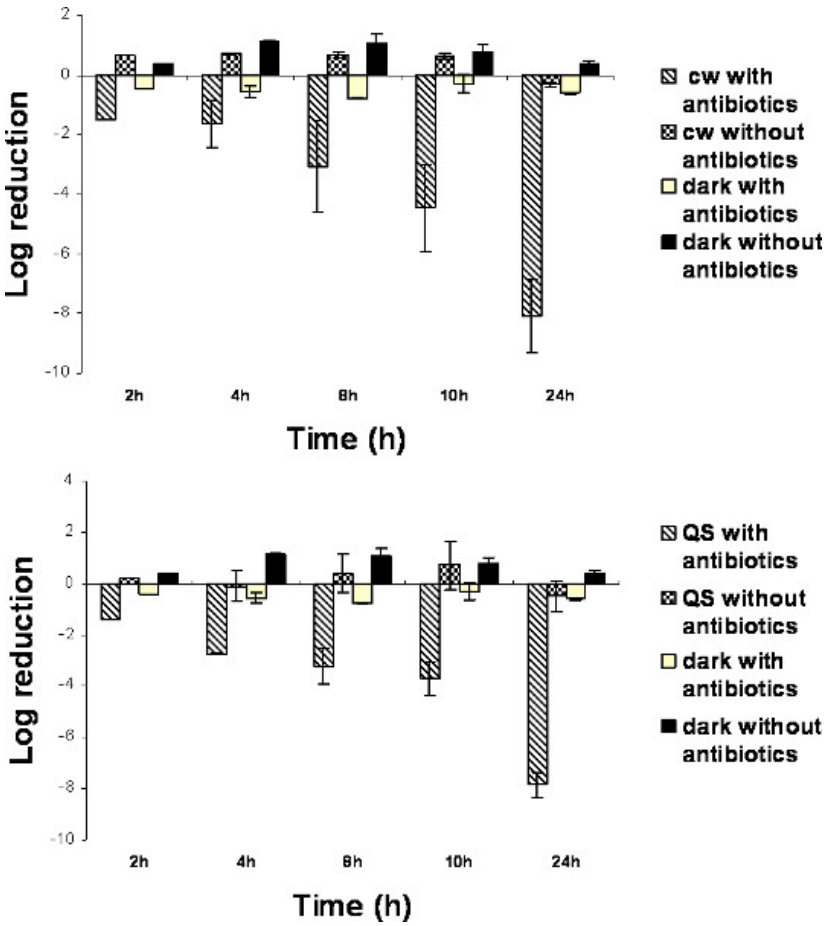

Figure 2. (a). Effect of CW-laser and gentamycin on *P. aeruginosa*. Samples were illuminated with a CW laser light (power of 100 mW) with and without gentamycin (50 µg/ml). The average of 3 experiments is presented. **(b)**. Effect of Q-switched laser and gentamycin on *P. aeruginosa*. Samples were illuminated with Q-switched laser light (1.65 MW) with and without gentamycin (50 µg/ml) on *P. aeruginosa* viability. The average of 3 experiments is presented.

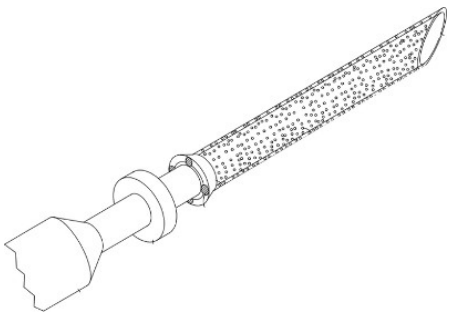

Figure 3. The proposed catheter based device for the biomedical treatment against *P. aeruginosa*. The dots in the figure represent light scattering points causing the light to be diffused into the treated tissue.

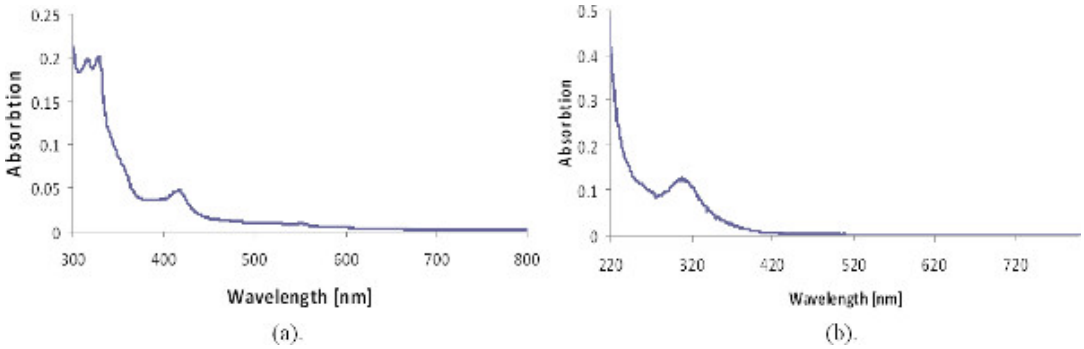

Figure 4. The absorption spectrum (in a.u.) around wavelength of 532 nm for: **(a)**. The bacteria, **(b)**. The gentamycin. Click here to view larger figure.

Discussion

Phototherapy has been a field of advanced multidisciplinary research in recent years emerging as a promising approach for treatment numerous diseases. In this context the use of light in the visible range has been extensively studied. For example, it has been found that infected wounds can be healed more effectively by exposing them to intense visible light for sterilization purposes. The mechanism of action for this approach was proven to be through the induction of light-induced oxygen radicals (ROS) which kill the bacteria[11].

Previous studies[6] have demonstrated much higher amounts of ROS in bacteria illuminated with blue light than those induced by red and near infra red light. This explains why most evidence in the literature concentrates on the bactericidal effect of blue light.

Another recent example for the use of laser light to fight resistant bacteria was demonstrated by Krespi *et al.*[12]. In that study laser generated shockwave technology was utilized to eradicate biofilms. By using a miniature Q-switched Nd:YAG laser and thin fibers, special probes generated plasma formation which produced shockwave effect. The authors showed that this method was able to effectively disrupt *P. aeruginosa* biofilms *in vitro*.

The approach we have presented in this study was somewhat different[13] as we attempted to increase the efficacy of non-photosensitizer antimicrobial agent using laser light. Our results suggest that by combining antibiotic treatment with illumination, the antimicrobial activity can be dramatically increased.

In fact, from a completely resistant phenotype, observed in the antibiotic alone the bacteria became sensitive in the presence of antibiotic and light treatment. The mechanism of action of this effect is not clear and will require further investigation. However, in the Electron-Paramagnetic Resonance (EPR) measurements that we have performed to examine whether ROS are generated during the treatment, no significant difference between the different treatments was obtained[13]. These results suggest that the effect of the combined treatment does not involve ROS production and different mechanisms have to be considered. It can be hypothesized that the light treatment changes the membrane permeability and eventually allows the antibiotics to penetrate into the bacteria cell yielding its killing.

Although the mechanism of operation is not fully explored, our approach highlight the potential of combined therapy of light with commercial antibiotic that may have been discarded due to antimicrobial resistance while by applying this combination can now be reused effectively in clinics.

Obviously as noted in the manuscript, illumination of several hours is needed in order to enhance the efficacy of the antibiotics. This is indeed a drawback of the proposed approach. The realization of such an illumination can be obtained *e.g.* by installing the illumination source inside catheters (as proposed by **Figure 3**). In addition to that, if the wound is external special banding as plaster with illumination source can be put on top of the wound and illuminate it for several hours *e.g.* while the patient is sleeping at night. If the infection is internal and the patient is hospitalized and he/she is connected to infusion bag for several hours, for some organs an illumination channel such as an endoscope or a special fiber can be approached to the organ and constantly illuminate it (with the applied antibiotic treatment) while the patient is hospitalized

(exactly as the infusion bag is connected to the patient for many hours). We fully agree that the approach is not good for treatment of generally infected organs.

Note that in this manuscript we show the advantage of the proposed technique for fast and practical application but in order to achieve this other studies are necessary such as *in vivo* experiments. The toxicity study on fibroblast or epithelial cells will be useful as well as the studies that will demonstrate the mechanism of the proposed therapy in bacterial cells are required. In addition in this paper we have hypothesized that light induced changes of the bacterial membrane that made it more permeable to the antibiotic. Obviously things will be different in a clinical setting where bacterial infections are due to biofilms. Then there are two major problems: Biofilm bacteria will be more resistant compared with their planktonic counterparts and penetration of antimicrobials in the biofilm mass will be limited. Therefore, exploring the effects of light and gentamycin on a *P. aeruginosa* biofilm model is the aim of our future study.

Disclosures

No conflicts of interest declared.

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