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ORIGINAL ARTICLE

The role of reactive oxygen species in the antibacterial photodynamic treatment: photoinactivation *vs* proliferation

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Significance and Impact of the Study: The main purpose in antibacterial photodynamic therapy (PDT) is to kill the micro-organisms that cannot be destroyed by conventional methods. Low-level light and/or low concentration of reactive oxygen species may trigger some biochemical pathways that lead to cell proliferation. Thus, there is a risk of bacterial cell proliferation during PDT. In this study we report that PDT with ICG application can induce biostimulation when laser dose and photosensitizer concentration are not optimized properly. Therefore, optimum dosimetry in PDT possesses great importance in the treatment of wounds infected by antibiotic-resistant bacteria.

Keywords
809-nm diode laser, antibacterial treatment, Gram-negative bacteria, indocyanine green, photoinactivation, proliferation.

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Abstract

Low-level light/low concentration of reactive oxygen species (ROS) may trigger some biochemical pathways that lead to cell proliferation. Thus, there is a risk of stimulation of bacterial cell proliferation during photodynamic therapy (PDT). In this study, PDT with different doses of 809-nm laser and indocyanine green (ICG) was investigated *in vitro* for safe bactericidal application. The combined effect of laser doses with ICG concentrations were examined on *Pseudomonas aeruginosa in vitro*. Data showed that low energy dose and ICG concentration caused bacterial cell proliferation. When these parameters were increased high enough, photoinactivation of the bacteria was achieved. Energy dose and photosensitizer concentration ranges at which proliferation, cell death or neither observed were determined. Furthermore, L-histidine was used as a scavenger of ROS to block the mechanism of biostimulation and cell killing. It inhibited proliferation when laser dose and ICG concentrations were low. It also inhibited cell killing when dose and concentration were high. Data showed that mechanisms of proliferation and cell killing depend on the amount of ROS and antibacterial photodynamic treatment have serious biostimulative risk. Effective range might need to be determined before any therapeutic usage. The risk seems to exist specifically at lower energy doses and photosensitizer concentrations.

Introduction

Photodynamic therapy (PDT) combines the use of light-sensitive drug (photosensitizer), which is nontoxic, with a suitable wavelength to activate the photosensitizer. Activated photosensitizers cause lethal damage to the target (Hamblin and Hasan 2004; Huang 2005; Maisch 2007; Wilson and Patterson 2008). The cytotoxicity of the photosensitizer depends on the dose of light and amount of oxygen available in the environment. The light absorbed by photosensitizer results in the transfer of energy to molecular oxygen yielding highly reactive oxygen species

(ROS) (Hamblin and Hasan 2004; Wilson and Patterson 2008; Vatansever *et al.* 2013). It has been shown before that cellular responses to ROS can cause cytotoxicity or enhanced cell survival depending on the concentrations. Higher concentrations are toxic and result in cell death, whereas lower concentrations may stimulate some biochemical pathways that induce cell survival (Wang *et al.* 2000; Klotz *et al.* 2003; Zhuang and Kochevar 2003; Bozkulak *et al.* 2007; Vatansever *et al.* 2013).

Photodynamic therapy is a widely investigated tool to treat mainly oncological and some nononcological diseases such as macular degeneration (Wilson and Patterson 2008). Recently antibacterial potential of PDT was revisited, since the life-threatening pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* became resistant to almost all known antibiotics. Many *in vitro* and *in vivo* studies were reported about antibacterial PDT applications on wounds that were infected by multidrug-resistant bacteria (Hamblin and Hasan 2004; Oriordan *et al.* 2005; Maisch 2007; Vatansever *et al.* 2013).

Indocyanine green (ICG) and 809-nm laser light combination is one of the new antibacterial PDT applications. Wavelengths around 800-nm are advantageous for biological applications, since they have deeper penetration capability in biological tissue than the wavelengths of the visible spectrum. So, using 809-nm light source with PDT applications gives opportunity to treat deeper infections or tumour cells, which are located deep inside the tissue. The appropriate photosensitizer for 809-nm wavelength is ICG which is an anionic water-soluble dye approved by the Food and Drug Administration (FDA). It has almost no toxicity and is commonly used for imaging purposes in clinics (Genina *et al.* 2004; Mamoon *et al.* 2009). There are two *in vitro* studies that investigated the antibacterial activity of PDT using ICG (Omar *et al.* 2008; Topaloglu *et al.* 2013). Our group has studied the antibacterial effect of the ICG-PDT with 809-nm diode laser on *Ps. aeruginosa* and *Staph. aureus in vitro* and reported positive results recently. In this earlier study we had found that the optimum parameters for at least 99% killing of wild-type strain of *Ps. aeruginosa* as 125 μg ml^{-1} ICG and 252 J cm^{-2} of light dose. During this study, the temperature was recorded with an infrared thermometer. No temperature increase was recorded. These measurements also showed that no photothermal mechanism was taking place in that bactericidal effect. Thus, it was concluded that 809-nm laser and ICG combination yielded a photodynamic effect without any temperature increase (Omar *et al.* 2008; Topaloglu *et al.* 2013).

However, during the predosimetry studies, we observed some unexpected results that raised the question whether the lower doses of application might cause bacterial proliferation instead of bactericidal effect. If the dosimetry of PDT was not optimized properly, not only photoinactivation of bacteria might fail but also proliferation of the pathogens could be observed. This might be the result of biostimulative effect of laser or laser-photosensitizer combination.

In this study we report for the first time that ICG-PDT application can induce biostimulation when laser dose and photosensitizer concentration are not optimized properly. In addition, the mechanism of biostimulation was investigated by using L-histidine as a scavenger of singlet oxygen.

Results and discussion

Antibacterial/biostimulative effect of PDT with different ICG concentrations and laser dose

Initially the effect of 84 J cm^{-2} of energy dose was investigated with seven different ICG concentrations (20, 50, 100, 125, 150, 200 and 250 μg ml^{-1}). Laser energy dose of 84 J cm^{-2} caused around 15% increase in cell number when applied alone. 84 J cm^{-2} of light with 20, 50, 100, 125 and 150 μg ml^{-1} of ICG resulted in statistically significant amount of cell proliferation in all of the ICG doses tested (61%, 26%, 34%, 61% and 38% respectively) ($P < 0{\cdot}05$). Increasing ICG concentration further to 200 and 250 μg ml^{-1} caused 21% and 29% of cell death in these PDT groups and it was not the desired bactericidal effect of PDT (Table 1).

When 168 J cm^{-2} of energy dose was applied alone, around 12% decrease was observed in bacterial burden. Then this energy dose was tested with the same ICG concentrations (Table 1). An energy dose of 168 J cm^{-2} with

Table 1 Effect of different indocyanine green (ICG)-photodynamic therapy (PDT) doses on percentage change in bacterial cell amount

	No Laser	Laser 84	Laser 168	Laser 252
No ICG	0.03%	**15%**	**−12%**	−0.12%
ICG 20	**48.90%**	**61%**	**12%**	**41%**
ICG 50	−4.95%	**26%**	**−0.15%**	3%
ICG 100	**−17%**	**34%**	**−99.49%**	**−37%**
ICG 125	−9%	**61%**	**−99.99%**	**−99.80%**
ICG 150	**−9.50%**	**38%**	**−99.98%**	**−99.99%**
ICG 200	−1.20%	**−21%**	**−99.99%**	–
ICG 250	**25%**	**−29%**	–	–

Colours show an increase or decrease of the bacteria population; yellow indicates the proliferative effect, light blue shows slight decrease in bacterial load and dark blue shows the antibacterial effect. Viability of *Pseudomonas aeruginosa* ATCC 27853 was determined after Laser only, ICG only and PDT applications. Bold numbers shows statistically significant groups ($P < 0{\cdot}05$) according to the untreated control group. (Light dose: 84, 168 and 252 J cm^{-2} and ICG concentrations: 20, 50, 100, 125, 150, 200 and 250 μg ml^{-1}).

20 μg ml^{-1} of ICG caused around 12% increase in viable cell count. When 50 μg ml^{-1} of ICG was used with 168 J cm^{-2} of energy dose, there was almost no change in the number of bacteria compared to the untreated control group. By increasing the ICG concentration, photoinactivation of bacteria was observed starting from the concentration of 100 μg ml^{-1} (Table 1). Towards the end of this ICG concentration range, the desired PDT effect was achieved by killing more than 99% of bacterial load.

Finally, 252 J cm^{-2} of energy dose was applied with the same ICG concentrations. This energy dose did not cause any significant change in the viable cell count when applied alone. With this energy dose, 20 μg ml^{-1} of ICG had a proliferative effect with about 41% increase on bacterial cell count. 50 μg ml^{-1} of ICG had no effect on viable bacteria; it was nearly same as the untreated control group. Starting from 100 μg ml^{-1} of ICG, bactericidal effect of PDT was observed in these groups. A total of 150 μg ml^{-1} of ICG was efficient enough to kill more than 99% bacterial load (Table 1).

Effect of L-histidine on the mechanism of PDT with different ICG concentrations and Laser dose

L-histidine was used as a scavenger of ROS. When it was used with laser energy dose and ICG concentrations which had proliferative effect on bacterial cells, amount of cells remained the same, no biostimulation or cell killing was observed (Fig. 1). It abrogated the biostimulative effect of the applied laser dose and ICG concentration.

When L-histidine was used with a higher ICG concentration at higher energy doses, we observed the exact opposite effect of antibacterial activity. A laser dose of 168 J cm^{-2} together with 125 μg ml^{-1} of ICG had a bactericidal effect on bacterial cells. However, when L-histidine was used, more than 50% increase was observed in the amount of bacterial viability. A laser dose of 252 J cm^{-2} with 125 μg ml^{-1} of ICG had a similar bactericidal effect. When they were used with L-histidine, it abolished the antibacterial activity of PDT and amount of cell population remained same as the control (Fig. 2).

One of the proposed mechanisms of PDT with ICG and wavelength around 800-nm light is the photochemical mechanism, namely the photo-oxidative effect. Szeimies and his colleagues investigated photo-oxidative killing effect of ICG and infrared light on human colonic cancer cells. They showed that ICG with infrared light can kill cancer cells via oxygen radicals and they proved this mechanism by using quenchers of singlet oxygen. When histidine and sodium azide, which are quenchers of singlet oxygen, were used, killing efficiency of PDT decreased. On the other hand using an extra singlet oxygen source (D_20) increased the photo-oxidative effect on cancer cells (Bäumler *et al.* 1999).

The efficacy of PDT mainly depends on light and drug dose as well as the time interval between drug addition

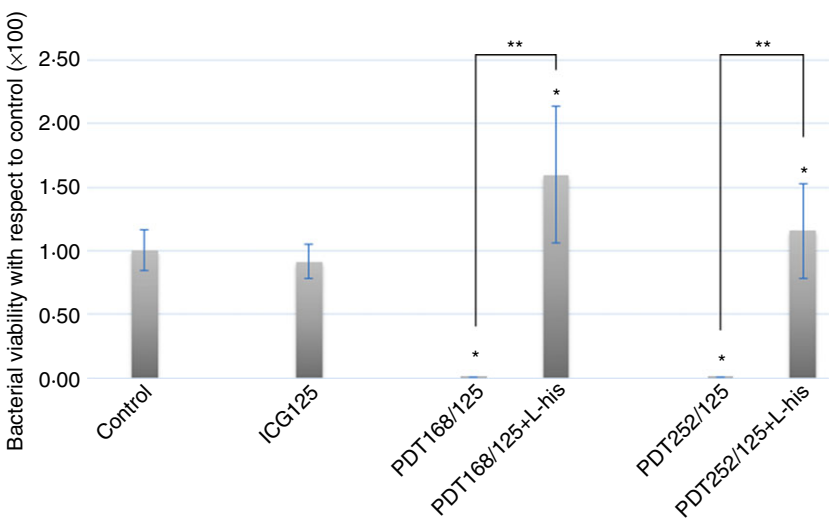

Figure 1 Bacterial viability percentage after Laser, photodynamic therapy (PDT) and PDT+L-histidine applications on *Pseudomonas aeruginosa* ATCC 27853. Viability of *Ps. aeruginosa* ATCC 27853 was determined after Laser only, PDT and PDT with L-histidine applications. Cell count in each experimental group was normalized with the untreated control group (L-histidine concentration: 50 mmol l^{-1}, Light dose: 84 J cm^{-2} and indocyanine green (ICG) concentrations: 20, 50, 100, 125, and 150 μg ml^{-1}). Each column indicates normalized data ± standard deviation ($n > 8$). *indicates the statistical significance in comparison to the untreated control group ($P < 0{\cdot}05$). **indicates the statistical significance between PDT and PDT+L-histidine groups ($P < 0{\cdot}05$).

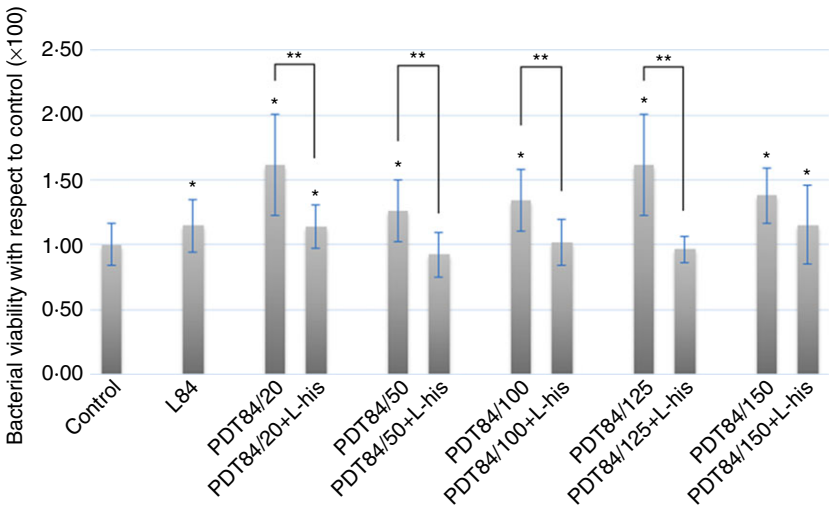

Figure 2 Bacterial viability percentage after indocyanine green (ICG), photodynamic therapy (PDT) and PDT+L-histidine applications on *Pseudomonas aeruginosa* ATCC 27853. Viability of *Ps. aeruginosa* ATCC 27853 was determined after ICG only, PDT and PDT with L-histidine applications. Cell count in each experimental group was normalized with the untreated control group (L-histidine concentration: 50 mmol l^{-1}, Light dose: 168 and 252 J cm^{-2} and ICG concentrations: 125 μg ml^{-1}). Each column indicates normalized data ± standard deviation ($n > 8$). *indicates the statistical significance in comparison to the untreated control group ($P < 0{\cdot}05$). **indicates the statistical significance between PDT and PDT+L-histidine groups ($P < 0{\cdot}05$).

and light application. When sufficient incubation time is allowed, ROS are produced as a result of the interaction between the light and drug. These ROS are responsible for the cell death. The amount of ROS generated depends on the concentration of drug, light dose and the amount of molecular oxygen available in the environment (Hamblin and Hasan 2004; Huang 2005; Maisch 2007; Wilson and Patterson 2008). When the drug and light doses are kept low, the amount of ROS will be low too. Therefore their destroying effect is expected to be low (3). However, lower concentration of ROS can stimulate different biochemical pathways in the cells causing cell survival and proliferation (Imlay and Linn 1987; Thomas *et al.* 1994; Miller and Britigan 1997; Davies 1999; Ruiz-Gines 2000; Palma *et al.* 2004; Salunkhe *et al.* 2005; Bartosz 2009). The two distinct effects of laser applications were studied in independent tracts in the literature, that is, proliferation effect for the cells (Karu *et al.* 1995) or inhibitory/killing effect for unwanted cancer cells (Tseng *et al.* 2003; Almeida *et al.* 2004; Crescenzi *et al.* 2004). This is the first time; these effects were studied in a continuum of dosimetry for PDT. Laser-ICG combination showed different qualitative effects depending on the quantitative changes of the doses applied. In the present study, it was observed that lower concentration of ICG with lower light dose caused bacterial proliferation instead of cell death. When we increased the light dose, the expected photoinactivation of bacteria could happen with lower doses of ICG. These results not only emphasized the significance of the PDT dosimetry but also showed the existence of a safety region. It should be optimized specifically for each bacterial strain used. In this study our results showed that >168 J cm^{-2} laser irradiation and higher than 100 μg ml^{-1} ICG should be combined in order to yield an efficient photoinactivation of *Ps. aeruginosa* ATCC 27853. Combination of ICG with <100 μg ml^{-1} and laser irradiation of <168 J cm^{-2} results in a proliferative effect on bacteria; we can refer to this situation as biostimulation.

To understand the mechanism of these applications and to relate the outcomes to the level of ROS, L-histidine was used together with ICG and near-infrared light. PDT group of 84 J cm^{-2} laser dose with different ICG concentrations caused significant increase in viable cell count. When L-histidine was added to these groups, proliferation was inhibited; the number of the cells remained same as the control. So it was thought that L-histidine quenched the already low amounts of ROS and consequently diminished its biostimulative effect. Later its effect was examined with higher ICG concentration and higher laser doses that have high antibacterial effect on bacteria. When L-histidine was used with following groups, 168 J cm^{-2} and 252 J cm^{-2} of laser dose with 125 μg ml^{-1} of ICG failed to induce photoinactivation. Instead of cell killing, proliferation of bacteria was observed when 168 J cm^{-2} of energy dose was used. This effect is thought to be originated from the fact that L-histidine quenched some of the ROS produced due to photodynamic activation, but the remaining amount of ROS might be enough to induce proliferation. 252 J cm^{-2} of laser dose together with 125 μg ml^{-1} of ICG failed to kill

the bacteria when L-histidine was added to this application. Concentration of the bacteria remained the same, this data suggests that all of the ROS products were captured by L-histidine and did not have any proliferative or killing effect on bacterial cells. Our data from the experiments with L-histidine proved that the antibacterial photodynamic action of near-infrared light and ICG is not photothermal, it is photo-oxidative damage due to the production of ROS. In addition, our results showed that proliferative risk of PDT with lower energy dose and photosensitizer concentrations is due to the amount of ROS which induce cell survival mechanisms.

Experiments showed that combination of laser with photosensitizer could result in photoinactivation if the doses of irradiation and concentration of ICG were selected in a specific range; otherwise PDT application could result in the proliferation of bacteria. Thus, both parameters were represented on Table 1 in order to visualize the ranges of the dosimetry for safe bactericidal effect and biostimulative effect against *Ps. aeruginosa*.

Materials and methods

Bacteria

Gram-negative bacteria *Ps. aeruginosa* ATCC 27853 (Gazi University, Ankara, Turkey) was used in this *in vitro* study. Single colony was inoculated into tryptic soy broth and incubated overnight at 37°C. Bacterial cells were harvested by centrifugation for 10 min at 4°C and the pellet was resuspended in phosphate buffered saline (PBS) to approximately 10^8–10^9 colony-forming unit mL^{-1} (CFU mL^{-1}).

Chemicals

ICG (Pulsion Medical System AG, Munich, Germany) was used as a photosensitizer and it was prepared in PBS before each experiment and all further procedures were performed in the dark. ICG concentrations used in this study were between 20 and 250 μg mL^{-1}. Absorption and fluorescence maxima of ICG are around 800 nm.

L-histidine (Merck KGaA, Darmstadt, Germany) was used as a scavenger of ROS. It was dissolved in PBS and its concentration was adjusted to 50 mmol l^{-1} when it was mixed with bacterial suspension and ICG solution.

Laser

An 809-nm diode laser was used as a light source. This laser was built in Bogazici University, Biomedical Engineering Institute, Biophotonics Laboratory (Geldi *et al.* 2006). The light dose was adjusted to 1·4 W cm^{-2} in each experiment. Laser power was checked with an optical powermeter (Newport 1918-C, Irvine, CA). The energy doses (84, 168 and 252 J cm^{-2}) were applied by increasing the exposure duration (60, 120 and 180 s respectively).

Study design

Experimental groups used in this study were shown in Table 2. In the ICG and PDT groups, 50 μl of ICG with a specific concentration was mixed with 50 μl of bacterial suspension in the wells of a 96-well plate. After addition of ICG, the wells were incubated in the dark for 15 min. In the Laser and Control groups, the bacterial suspension in the wells was mixed with equal volume of PBS (50 μl). Then the bacterial suspension in the PDT and Laser groups was irradiated by laser. Following irradiation, viable cell counts were determined by serial dilution method. All diluted samples were plated on tryptic soy agar. After a 24-h incubation period, colony-forming units were counted to determine viable bacteria after each application. All experiments were repeated at least three times, and all conditions were done in triplicates within each experiment.

Effect of L-histidine on the mechanism of PDT

L-histidine solution was added to selected PDT groups to assess the effect of ROS production. After bacterial cell suspension was incubated with ICG solution for 15 min, 50 μl of L-histidine solution was added and this mixture was incubated for additional 15 min before laser irradiation. These groups were incubated with different ICG concentrations and irradiated with different laser doses in the presence of L-histidine at a final concentration of 50 mmol l^{-1} (Table 2). Viable cell counts after applications were determined as described above.

Statistical analysis

In each experiment, some of the wells were used for experimental groups and others were assigned to the control samples. In order to keep the conditions constant,

Table 2 Experimental groups

	Laser	ICG	L-histidine
Control	−	−	−
ICG*	−	+	−
Laser†	+	−	−
PDT	+	+	−
PDT+L-histidine‡	+	+	+

ICG, indocyanine green; PDT, photodynamic therapy.
*ICG concentrations: 20, 50, 100, 125, 150, 200 and 250 μg ml^{-1}.
†Laser energy doses: 84, 168, and 252 J cm^{-2}.
‡L-histidine concentration: 50 mmol l^{-1}.

viable cell counts determined after the serial dilution method were normalized by taking the ratio with corresponding control groups on each 96-well plate. Normalized data were analysed for statistical significance with one-way ANOVA and two-tailed Student's *t*-test. The results were considered significant when P value was $<0{\cdot}05$.

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Conflict of Interest

No conflict of interest declared.

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