ANTIBACTERIAL PHOTODYNAMIC THERAPY WITH INDOCYANINE GREEN AND NEAR-INFRARED LIGHT

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ABSTRACT

ANTIBACTERIAL PHOTODYNAMIC THERAPY WITH INDOCYANINE GREEN AND NEAR-INFRARED LIGHT

Increase in antibiotic-resistance is a worldwide health problem which may result in septicemia and subsequent death in recent years. Some of these deaths are caused by nosocomial, burn or chronic wound infections. Photodynamic therapy can be an alternative technique in treatment of infections. This research aimed to investigate the bactericidal effect of photodynamic therapy with indocyanine green and near-infrared light *in vitro* and *in vivo*.

First, the effect of indocyanine green and 809-nm laser light was examined on wild type and resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro*. Indocyanine green concentration and laser dose were initially optimized for wild type strains. After determining most effective concentrations with specified light dose, they were applied on resistant strains. This method was totally efficient to kill these strains and optimum doses varied with different strains. Later, this method was examined on rat excisional and abrasion wound models. Wounds were infected by resistant strains of *S. aureus* and *P. aeruginosa*. Optimum parameters could not be found for excisional wounds because of bleeding, but infected abrasion wounds could be successfully treated. Around 90% reduction in bacterial burden was observed. Applied energy dose did not cause any thermal damage on healthy tissue.

This study showed that indocyanine green together with near-infrared light might be a promising antibacterial method to eliminate infections in clinics and accelerate wound healing process.

Keywords: Photodynamic Therapy, Near-infrared Light, Indocyanine Green, Antibacterial, Wound Infections, *Staphylococcus aureus*, *Pseudomonas aeruginosa*.

ÖZET

İNDOSİYANİN YEŞİL VE YAKIN-KIZILALTI IŞIK İLE ANTİBAKTERİYEL FOTODİNAMİK TERAPİ

Son yıllarda antibiyotik direnci geliştiren patojenlerdeki artış, septisemi ve ölümle sonuçlanan bir sağlık sorunu haline gelmiştir. Septisemiye bağlı ölümlerin bazıları hastane, yanık ya da kronik yara enfeksiyonlarından kaynaklanmaktadır. Fotodinamik tedavi lokal enfeksiyonların tedavisinde alternatif bir yöntem olabilir. Bu araştırmada amaçlanan yakın-kızılaltı ışık ile indosiyanin yeşil kullanarak fotodinamik terapinin antibakteriyel etkisini *in vitro* ve *in vivo* ortamda araştırmaktır.

İlk olarak, indosiyanin yeşil ve 809-nm laserin etkisi yabanıl ve dirençli *Staphylococcus aureus* ve *Pseudomonas aeruginosa* suşları üzerinde *in vitro* ortamda incelenmiştir. Yabanıl suşları yeterli miktarda yok eden dozlar belirlendikten sonra, bu dozlar dirençli suşlara uygulanmıştır. Bu yöntemin yabanıl ve dirençli suşları yok etmede etkili olduğu ve yeterli ilaç/ışık dozlarının türe bağlı olarak değiştiği anlaşılmıştır. Daha sonra, bu yöntem sıçan eksizyon ve abrasyon yara modelleri üzerinde incelenmiştir. Yaralar dirençli suşlar ile enfekte edildikten sonra ideal ilaç konsantrasyonu ve ışıma dozu araştırılmıştır. Oluşturulan eksizyon yaralarında enfeksiyonu yok edecek yeterli parametreler bulunamamıştır. Ancak enfekte edilen abrasyon yara modeli başarıyla tedavi edilebilmiş ve canlı bakteri sayısı % 90 oranında azaltılmıştır. Uygulanan ışıma dozu hedef doku ve çevresinde herhangi bir ısıl harabiyete sebep olmamıştır.

Bu çalışma, yakın-kızılaltı ışıkla birlikte uygulanan indosiyanin yeşilin sıçanlarda yarı enfeksiyonlarını tedavi etmede ve yaraların iyileşmesini hızlandırmada umut verici bir antibakteriyel yöntem olduğunu göstermiştir.

Anahtar Sözcükler: Fotodinamik Terapi, Yakın Kızılaltı Işık, İndosiyanin Yeşil, Antibakteriyel, Yara Enfeksiyonları, *Staphylococcus aureus*, *Pseudomonas aeruginosa*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

°C	Degrees Celcius

LIST OF ABBREVIATIONS

PDT	Photodynamic therapy
PS	Photosensitizer
ICG	Indocyanine green
US FDA	United States Food and Drug administration
DNA	Deoxyribonucleic acid
ALA	Aminolevulinic acid
CFU	Colony forming unit
J	Joule
W	Watt
mW	milliWatt
IR	Infrared
NIR	Near-Infrared
μg	microgram
nm	nanometer
mm	millimeter
cm	centimeter
μm	micrometer
μl	microliter
ml	milliliter
PBS	Phosphate buffered saline
rpm	Revolutions per minute
mg	milligram
kg	kilogram
g	gram
v	Volume

1. INTRODUCTION

1.1 Motivation

Wound infections are considerably great global problem in last decades. These infections can cause morbidity and mortality. The pathogens, which cause lethal infections, have been cured conventionally by antibiotics. The rapidly increasing antibiotic resistance makes the treatment of infected wounds difficult and even impossible. Thus this situation has motivated researchers to concentrate on the development of novel antibacterial treatments [1, 2].

A promising novel treatment is photodynamic therapy (PDT). It involves the use of non-toxic dyes, which is called photosensitizers (PS) in combination with harmless light of the appropriate wavelength to excite the photosensitizer [3]. PDT has some advantages over other conventional treatments. Firstly, resistance can develop after repeated use of antibiotics. For PDT applications, it is unlikely to develop from repeated use. One of the features of antibacterial PDT is the lack of selection of photo-resistant strains after multiple treatments. Secondly, both the PS and the light are applied locally to the target tissue. There are many formulations allowing specific delivery of photosensitizer to the infected area. Therefore, it reduces the risk of adverse side effects. The probability of destroying indigenous bacteria, which are away from the application area, is very low. PDT also has small probability to promote the onset of mutagenicity. Superficial wound infections are more appropriate for the treatment by PDT due to their easy accessibility to both photosensitizer and light [4]. Due to its ease of application, being less expensive and having very less side effects compared to other conventional treatments, it is a good alternative for treatment of wound infections.

Photodynamic Therapy is not commonly used method in clinics. There are several parameters needs to be assessed and investigated such as power of the laser light, exposure duration, concentration of the photosensitizer, optical properties and

location of the target tissue [5].

Different wavelengths of lasers have been investigated for antibacterial Photodynamic Therapy, predominantly wavelengths in visible spectrum. But near infrared lasers have been used in very few *in vitro* studies, but not in *in vivo* or clinical studies. This study will show the sensitivity of gram-positive and gram-negative bacteria to lethal photosensitization of Indocyanine Green with near-infrared laser light, especially in animal model. Near-infrared laser light has more capacity to penetrate biological tissue than the light of visible wavelengths. Beside this, Indocyanine Green is water-soluble dye, which is approved by US FDA for medical applications [1]. Its toxicity is very low. Therefore, ICG-PDT has been used to treat tumors. But the only area of PDT applications for infections is the treatment of acne vulgaris [3]. Except this, ICG-PDT has not yet been used to treat infected wounds. This project will propose optimum parameters (power, exposure time, fluence, concentration) for clinical use of near-infrared laser (809-nm) in Photodynamic Therapy applications.

1.2 Objectives

1. To assess the main parameters (output power, energy dose, exposure duration, photosensitizer concentration) of Photodynamic Therapy with near-infrared laser and its appropriate photosensitizer (Indocyanine Green).

2. To find out the bactericidal effect of PDT with Indocyanine Green and near-infrared laser on different bacterial strains (*Staphylococcus aureus* strains and *Pseudomonas aeruginosa* strains) *in vitro*.

3. To investigate possible effect of antibiotic-resistivity of bacterial strains on antibacterial capacity of Photodynamic Therapy.

4. To assess the effect of output power difference during PDT application *in vitro*, in terms of heat generation and cell viability.

5. To find out the bactericidal effect of PDT with Indocyanine Green and near-

infrared laser on different infected wound models *in vivo*.

1.3 Outline

Background of wound infections and photodynamic therapy and literature survey is given in this chapter.

In chapter 2, *in vitro* research about optimum parameters for antibacterial PDT with near-infrared laser and ICG is explained. Determination of optimum laser energy dose and optimum ICG concentration to destroy efficiently wild and resistant types of *S. aureus* and *P. aeruginosa* strains, effect of Laser-alone and ICG-alone applications on bacterial cells are described in detail.

In chapter 3, study about the influence of different output powers while keeping laser energy dose constant by changing exposure duration is explained with an *in vitro* study on *P. aeruginosa* strain.

In chapter 4, an *in vitro* study about the risk of biostimulation during antibacterial PDT application on *P. aeruginosa* strain is explained.

In chapter 5, description of different infected wound models (excisional and abrasion) on rats is explained. Then the study about finding out optimum parameters of laser energy and ICG concentrations to treat infected wounds is given. Wound healing process is described by hematoxylene-eosin staining results, besides colony counting.

In chapter 6, final discussion and conclusion are given and overall study is evaluated.

2. BACKGROUND

2.1 Wound infections and Wound-infecting organisms

Chronic infections can be life-threatening diseases, which may result in delayed healing and/or mortality in further stages. Some examples are surgical wound infections, nosocomial infections, burn infections, soft-tissue infections, oral and dental infections, and other wound infections such as leg ulcers, abrasion wounds [2, 6].

Skin is the first barrier of the body. When it loses its integrity, tissues beneath it can be easily contaminated by pathogens. Thus burns, surgical wounds are highly susceptible to infections [2, 7]. Their treatment may be impossible if the patients suffer from diabetes. Healing process is slower in diabetes; infections make it more difficult [7]. Wound infecting organisms are bacteria, viruses and fungi. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* are the common bacteria, responsible for various bacterial wound infections. *Candida albicans* and *Trichophyton rubrum* strains are common cause of fungal infections. *Herpes simplex*, and human papilloma virus are most frequent viruses that are responsible for viral lesions [7, 8, 9, 10, 11, 12, 13, 14].

2.2 Conventional treatments of Wound Infections and Antibiotic-resistivity of Pathogens

The conventional treatment of pathogenic bacteria which cause lethal wound infections are antibiotics and the rapidly increasing resistivity to these drugs makes conventional method difficult and even impossible [9]. Changes in cellular mechanisms or mutations in DNA structure of pathogens make them more resistive to antibiotics. Chronic wound infections become untreatable because of increasing antibiotic-resistivity and result in bacteremia and septicemia, which lead to removal of tis-

sue/organ or even death [6].

Other antimicrobial agents such as topical antiseptics i.e. iodine-containing solutions, silver preparations or surgical removal can be used as an alternative to antibiotics. There is not any resistivity mechanism for these methods but still there are some limitations or disadvantages. They are not non-toxic and/or harmless. Generally they do not target only bacteria, but also normal cells [4, 15, 16, 17]. Some of them (e.g. hypochlorite) cause skin irritation. They kill keratinocytes, fibroblasts, too. They influence wound healing negatively. Systemic absorption of an antiseptic such as iodine compound can cause many adverse effects from psychological disorders to skin reactions or acidosis and also metabolic disorders such as hyperthyroidism. Severity of iodine solution application depends on the concentration of iodine absorbed by tissue. It is highly possible to absorb more iodine compound for wounds or burns. Therefore using iodine as an antiseptic on infected wounds or burns is not recommended [15, 16, 17]. Some silver preparations such as silver nitrate, silver sulfadiazine are used as antimicrobial agents, too. They are not capable to penetrate deep inside the tissue [16]. So those agents are not a good choice for deeply infected wounds or burns. Therefore these problems have motivated the researchers to concentrate on the development of novel, convenient and inexpensive anti-microbial treatment strategies for fighting pathogens and wound healing [2, 3, 8, 18, 19].

2.3 Photodynamic Therapy: Mechanism of Action

Photodynamic therapy (PDT) involves the use of non-toxic dye (photosensitizer) in combination with appropriate wavelength to excite the photosensitizer. Excited photosensitizers become the source of oxygen radicals by transferring their energies to molecular oxygen. These reactive oxygen species finally cause lethal damage to the target (Figure 2.1) [2, 18, 19, 20, 21].

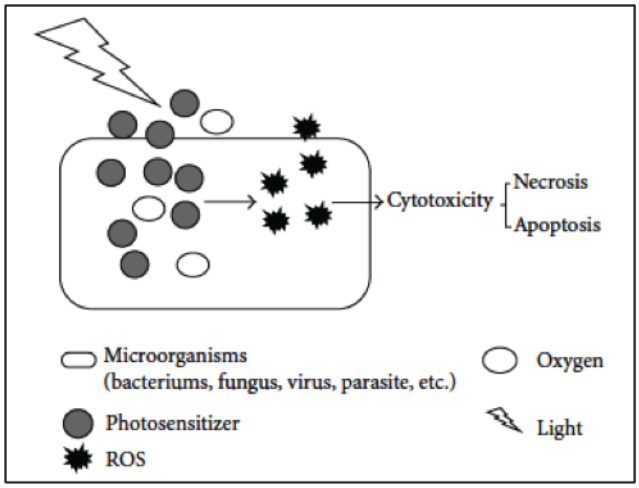

Figure 2.1 Simple schematic representation of Photodynamic Therapy [22].

There are two possible mechanism of photodynamic therapy. When photosensitizer absorbs light, it is excited to singlet state. Then excited photosensitizer in singlet state undergoes to triplet state, which is a lower energy, and longer-lived state. After this, transferred energy to the photosensitizer may be transferred to organic substrates, which are in the environment. This process is Type I mechanism of PDT. At the end of this energy transfer, radical ions are produced to react with oxygen molecule. As a result, cytotoxic species are produced (Figure 2.2) [3, 8, 18, 22, 23].

In Type II mechanism of PDT, the energy from light is transferred to molecular oxygen and it resulted in reactive oxygen intermediates at the end, such as reactive singlet oxygen, hydrogen peroxide or hydroxyl radical which can destroy biological molecules (proteins, nucleic acids) (Figure 2.2) [3, 8, 18, 22, 23].

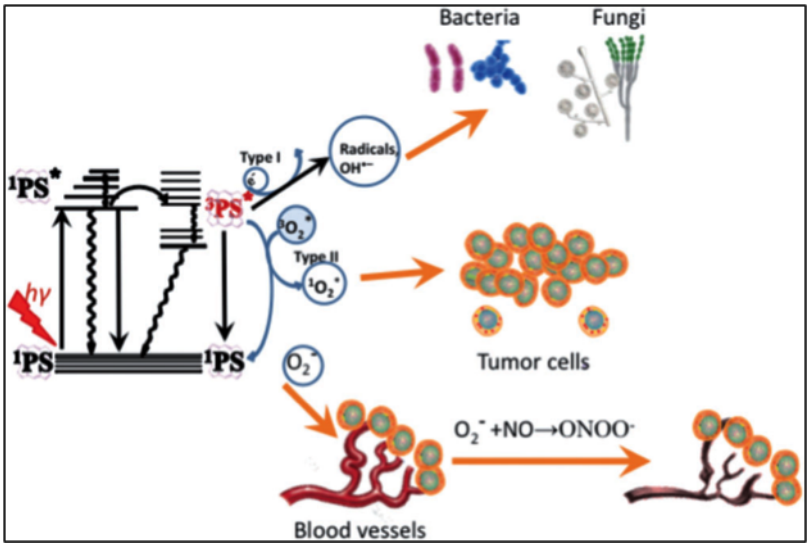

Figure 2.2 Schematic representation of type I and type II mechanisms of photodynamic therapy [24].

2.4 Light sources and Wavelengths

Wavelengths in visible and near-infrared spectrum are mostly preferred wavelengths for photodynamic therapy applications. There are many PDT studies using coherent or non-coherent light sources, such as incandescent lamps, xenon lamps, and lasers. It is thought that lasers that is coherent, is ideal for photodynamic purposes [23].

Light sources which emits light in visible spectrum are generally chosen many antibacterial and cancer studies. But visible light has limited penetration capacity in biological tissue. On the other hand, wavelengths in the range of near-infrared spectrum, such as 809-nm, have more capacity to penetrate biological tissue than rest of the spectrum. Penetration depth in biological tissue for the wavelengths around 650-nm is 3-3.5 mm, whereas for near-infrared light it reaches up to 6 mm (Figure 2.3) [25]. Thus infections or cancerous tissue spread to deep inside can be treated by near-infrared wavelengths of light in combination with appropriate photosensitizers.

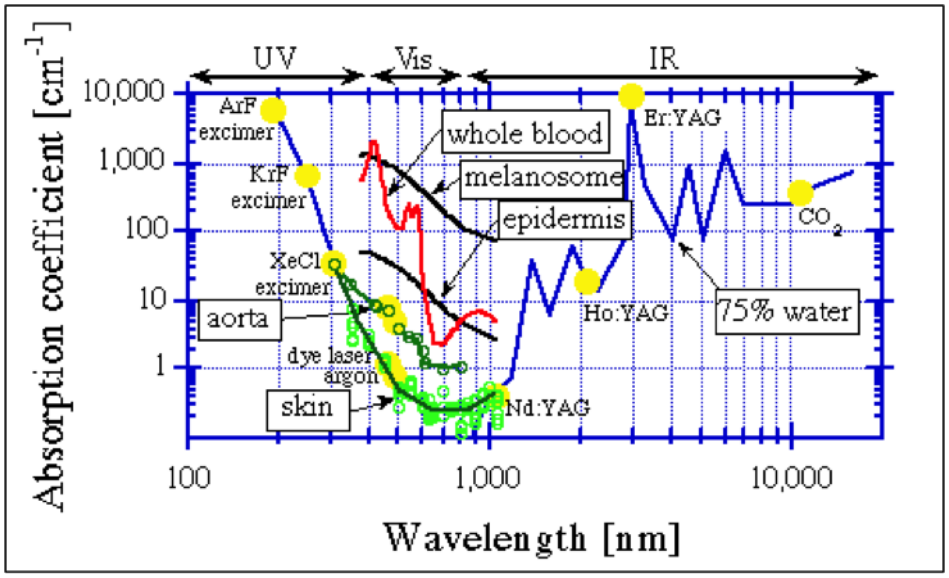

Figure 2.3 Primary absorption spectra of biological tissue (Lecture notes of ECE532 Biomedical Optics, Oregon Graduate Institute).

2.5 Photosensitizers

Mostly used photosensitizers are toluidine blue, methylene blue, chlorin(e6) conjugates, porphyrin and its derivatives, and ALA derivatives. They highly absorb visible light. These photosensitizers are advantageous for antibacterial photodynamic action because of their cationic nature, which is attractive to anionic structure of bacterial cell [26, 27, 28, 29].

Indocyanine green (ICG) is a specific photosensitizer for wavelengths in near-infrared spectrum with a high absorption around 800-nm (Figure 2.4). ICG is water-soluble, anionic tricarbocyanine dye with almost no toxicity and it is approved by United States Food and Drug Administration for medical applications to observe liver function, cardiac output and blood volume [30, 31, 32, 33]. Thus PDT with ICG has been investigated to treat tumors, e.g. pancreatic, lung, skin, colonic and breast tumors [34, 35, 36, 37] and for bactericidal purposes in the treatment of acne vulgaris [3, 38]. However, its anionic nature decreases its availability as an antibacterial agent.

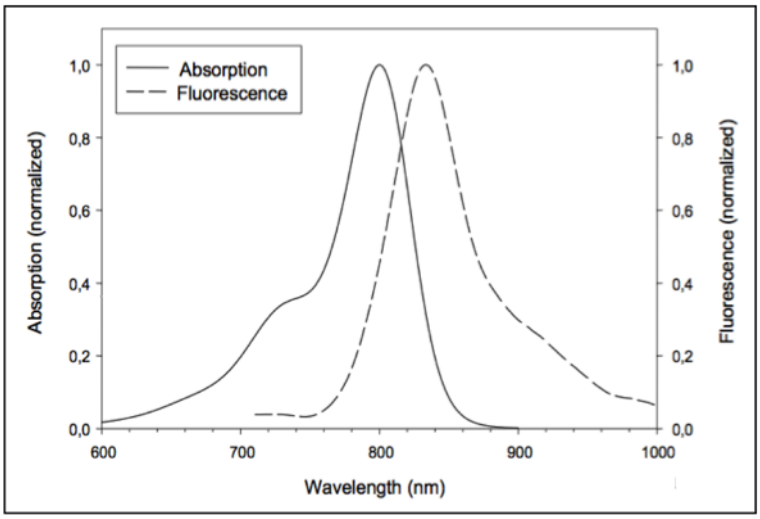

Figure 2.4 Absorption and fluorescence spectra of ICG (Documents from Pulsion Medical System).

2.6 Clinical applications of PDT

PDT has now been used as an approved clinical tool in many areas around the world. But its usage is still limited. Mostly preferred treatments in clinics are for treating cancer cells (head and neck cancers, skin cancers, etc.) and some other non-oncological diseases (age-related macular degeneration) [7, 20, 39, 40, 41] and there are some limited antibacterial applications for destroying microorganisms such as acne vulgaris [8, 12, 42, 43, 44]. It is still in promising situation offering a key role in clinics, especially for the treatment of cancerous tissue and infections.

2.7 Antibacterial photodynamic therapy

Photodynamic therapy had been used to destroy some microorganism at the early 1900's. After the discovery of penicillin at 1928, scientific world headed towards researchs on infection treatment with antibiotics, and PDT was disregarded as an antibacterial tool [3, 19, 45]. After the increase of antibiotic-resistivity of pathogens, recent *in vitro* studies showed the rich potential of PDT to treat wound infections

caused by antibiotic resistant bacteria [46, 47, 48].

Actually exact mechanism of antibacterial PDT is not known, but type II mechanism of PDT is accepted for antibacterial action. Reactive oxygen species produced during PDT are thought to react with bacterial cell or to go inside the cell and react with inner membrane structures [2, 8, 18]. Photoinactivation process of bacteria depends on the type of it. Gram-positive and gram-negative bacteria have different cell wall structure (Figure 2.5). Its interaction with photosensitizer and reactive oxygen species are different, too. The orientation of peptidoglycan layer and the additional membrane layer outside the peptidoglycan layer of gram-negative bacteria generally prevents photosensitizer to interact or go inside the cells [2, 3, 8, 18, 19, 45]. So they show resistivity to many kinds of photosensitizers, especially anionic ones. Anionic and neutral photosensitizer can easily bind and react with gram-positive bacteria, so they can be destroyed easily by photoinactivation. But photoinactivation of gram-negative bacteria is more difficult.

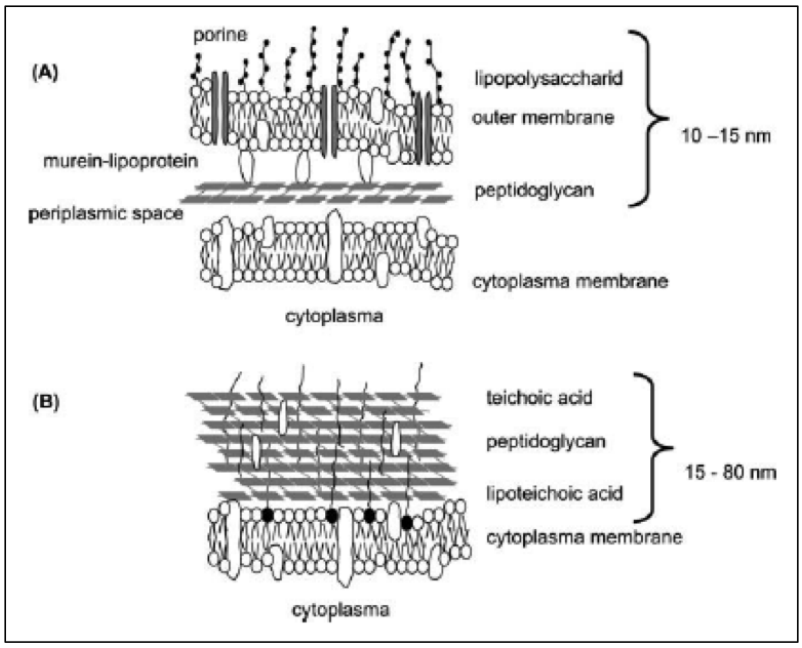

Figure 2.5 Schematic representations of (A) the arrangement of the cell walls of gram-negative and (B) gram-positive bacteria [3].

PDT has numerous advantages over antibiotics, antiseptics and surgical removal. Multidrug-resistivity can develop after repeated use of antibiotics. However, develop-

ment of photo-resistance after multiple treatments of antimicrobial PDT has not yet been reported [5, 49]. Due to its ease of application on the affected area, having much less side effects, being minimally invasive and being cheaper compared to antibiotics, it is a good alternative tool for treatment of wound infections [50].

3. ANTIMICROBIAL PDT ON WILD-TYPE AND RESISTANT STRAINS OF *S. AUREUS* AND *P. AERUGINOSA* WITH ICG AND 809-NM DIODE LASER: *IN VITRO* STUDY

3.1 Introduction

PDT with ICG has been investigated to treat tumors, e.g. pancreatic, lung, skin, colonic and breast tumors [34, 35, 36, 37] and for bactericidal purposes in the treatment of acne vulgaris [3, 38]. Besides limited clinical studies on the treatment of acne vulgaris [3, 38], bactericidal effect of PDT with ICG has recently been investigated in very few *in vitro* studies [9, 51]. Thus, there are several parameters needs to be investigated such as power and the energy dose of the laser light, exposure duration of the laser, concentration of the photosensitizer, optical properties and location of the diseased tissue.

The aim of this study was to investigate the effects of PDT with ICG on viability of wild type and resistant bacterial strains (*S. aureus strain* ATCC 25923, *S. aureus* clinical isolate 1755, *P. aeruginosa* strain ATCC 27853 and *P. aeruginosa strain* ATCC 19660) *in vitro*.

3.2 Materials and Methods

3.2.1 Bacterial strains

The bacterial strains used in this study were wild type *S. aureus* ATCC 25923 (Refik Saydam National Public Health Agency, Ankara, Turkey), resistant clinical isolate *S. aureus* 1755 (Gazi University, Ankara, Turkey), wild type *P. aeruginosa* ATCC

27853 (Gazi University, Ankara, Turkey) and resistant type *P. aeruginosa* ATCC 19660 (ATCC, USA). After overnight incubation in tyriptic soy broth (Merck KGaA, Darmstadt, Germany) at 37 °C, bacterial cells were harvested by centrifugation (3000 rpm for 10 minutes at 4°C) and the pellet was resuspended in phosphate buffered saline (PBS) to approximately 10^6-10^7 colony forming unit/ml (CFU/ml) for *S. aureus* strains and 10^8-10^9 CFU/ml for *P. aeruginosa* strains.

3.2.2 Photosensitizer

ICG (Pulsion Medical Systems AG, Munich, Germany) was used as a photosensitizer in this study. Absorption and fluorescence maximum of ICG are in the near-infrared range. Wavelength around 800-nm is suitable for this photosensitizer. Fresh stock solutions were prepared in PBS before each experiment and kept in the dark. ICG concentrations used for *S. aureus* strains were between 0.5-8 μg/ml and for *P. aeruginosa* strains were between 50-150 μg/ml.

3.2.3 Laser Light

A computer controlled 809-nm diode laser system was used for PDT experiments. This laser system was developed in Bogazici University, Biomedical Engineering Institute, Biophotonics Laboratory [52]. The distance of the laser probe to the plate surface was adjusted to apply 1.4 W/cm^2 in each experiment (Figure 3.1).

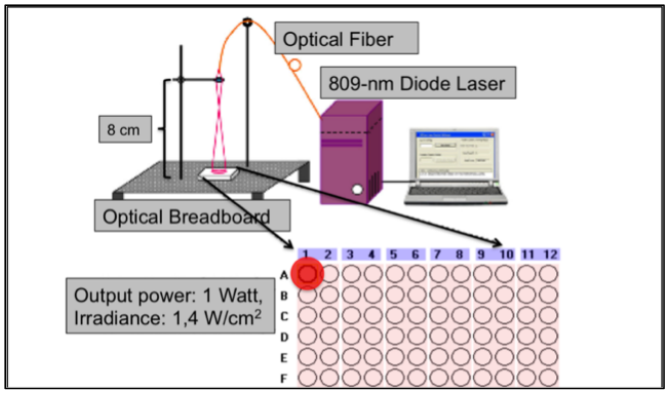

Figure 3.1 Laser Setup. 809-nm diode laser is a computer-controlled system. The optical fiber of the laser system was adjusted to apply 1.4 W/cm^2 on the plate surface. The distance between the laser probe and the plate surface was 8 cm.

Laser power was checked with an optical powermeter (Newport 1918-C, CA, USA). The energy doses between 80-420 J/cm^2 were applied by increasing the exposure duration from 60 seconds to 5 minutes.

3.2.4 Study Design

The effects of ICG-PDT with different ICG concentrations and different energy densities were studied *in vitro*. Following groups were tested:

1. **Control group:** no ICG, no light
2. **Laser-only group:** Irradiated without ICG
3. **ICG-only group:** Incubation only with ICG
4. **PDT (Laser + ICG) group:** Irradiated in the presence of ICG

50 μl aliquots of bacterial suspension were transferred into the wells of 96-well plate. In the ICG and PDT groups, 50 μl of ICG with a specific concentration was added to each well and mixed with bacteria. After addition of ICG, the wells were

incubated in dark for 15 minutes. 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 were irradiated. Following irradiation, bacterial suspension in all groups was diluted according to the serial dilution method. Then diluted samples were plated on tyriptic soy agar and incubated in dark for 24 hours. The number of colony forming units (CFU) were counted by naked eye and multiplied by dilution factor 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.

3.2.5 Optimization of laser light dose

The killing effect of laser light dose was investigated on *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 wild type strains as described above. Energy doses of 84 J/cm^2, 252 J/cm^2 and 420 J/cm^2 were applied on bacterial suspension of *S. aureus* changing the exposure duration. Energy doses of 84 J/cm^2, 168 J/cm^2 and 252 J/cm^2 were applied on *P. aeruginosa*. The temperature of the bacterial suspension in a well was measured with a noncontact infrared thermometer (ST Pro, Raytek Corporation, California, USA) immediately after each laser application. No temperature increase was recorded. Viable cell was counted by serial dilution method.

3.2.6 Effect of different ICG concentrations

The killing effect of ICG concentration was investigated on *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 wild type strains without applying light. For *S. aureus* ATCC 25923, 0.5, 1, 2, 4, 6, 8 μg/ml ICG were used. For *P. aeruginosa* ATCC 27853, 50, 100, 125, 150 μg/ml ICG were applied. The viability was determined by serial dilution method.

3.2.7 Dose estimation for ICG-PDT on wild-type strain of *S. aureus*

In the PDT groups of *S. aureus* ATCC 25923, bacterial suspensions were mixed with 6 different ICG concentrations (0.5, 1, 2, 4, 6, 8 μg/ml). After 15 minutes incubation with ICG, energy dose of 84 J/cm^2 was applied on the bacterial suspension. Immediately after the laser applications, bacterial suspensions were diluted to count viable bacteria.

3.2.8 Dose estimation for ICG-PDT on wild-type strain of *P. aeruginosa*

In the PDT groups of *P. aeruginosa* ATCC 27853, bacterial suspensions were mixed with the concentrations of 50, 100, 125, 150 μg/ml of ICG. After 15 minutes incubation with ICG, energy dose of 252 J/cm^2 was applied on the bacterial suspension.

3.2.9 ICG-PDT application on resistant strains of *S. aureus* and *P. aeruginosa*

Optimum parameters of ICG-PDT were determined with wild type strains of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853. Energy dose of 84 J/cm^2 and 4-6 μg/ml of ICG concentrations were applied on the resistant strain of *S. aureus* 1755. Energy dose of 252 J/cm^2 and 100-125 μg/ml of ICG concentrations were applied on resistant strain of *P. aeruginosa* ATCC 19660.

3.2.10 Data and Statistical analysis

In each experiment, some of the wells were used for experimental groups and others were assigned to control samples. In order to keep the conditions constant, viable cell counts determined after serial dilution method were normalized by taking the ratio with corresponding control groups on each 96-well plate. Normalized data were

analyzed for statistical significance with one-way ANOVA and two-tailed Student?s t-test. The results were considered significant when the p value was less than 0.05.

3.3 Results

3.3.1 Effect of laser light only

3 Different light doses were applied both on *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 to determine the effect of laser light on bacterial viability. Even the highest doses (420 J/cm^2 for *S. aureus* and 252 J/cm^2 for *P. aeruginosa* strains) tested did not cause significant cell death as expected. Figure 3.2, 3.3, 3.4 and 3.5 shows the results of cell viability for every bacterial strain after the application of their corresponding energy dose.

3.3.2 Effect of ICG concentrations on bacterial viability

In order to find the safe working range for ICG, 6 different ICG concentrations (0.5, 1, 2, 4, 6, 8 μg/ml) were tested on *S. aureus* ATCC 25923 and 4 different ICG concentrations (50, 100, 125, 150 μg/ml) were tested on *P. aeruginosa* ATCC 27853. Among the concentrations used in this study ICG induced cytotoxicity was not observed.

3.3.3 Dose estimation for ICG-PDT on wild-type strain of *S. aureus*

The lowest light dose (84 J/cm^2) was used with previously tested ICG concentrations (0.5, 1, 2, 4, 6, 8 μg/ml) on *S. aureus* ATCC 25923. In the PDT groups, all of the applications caused significant decrease on the cell viability. More than 90% decrease with the application of 84 J/cm^2 of light dose and 4 μg/ml of ICG concentration was observed. When the concentration of ICG was increased (6 and 8 μg/ml) in the

PDT group almost 100% cell death was achieved (Figure 3.2).

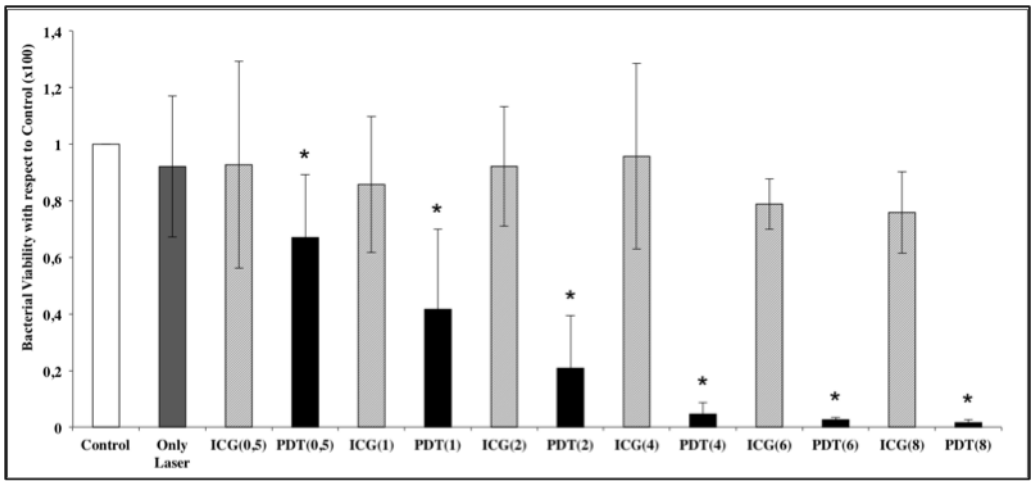

Figure 3.2 Effect of ICG, Laser and PDT applications on wild type *S. aureus* ATCC 25923. Cell viability of *S. aureus* ATCC 25923 after Laser only, ICG only and PDT applications were determined by viable cell count as described above. Bacterial cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2 and ICG concentrations: 0.5, 1, 2, 4, 6, 8 μg/ml). Each column indicates normalized data $\pm$ standard deviation (n>8). $*$ indicates the statistical significance (p<0.05) in comparison to the untreated control group.

According to these results, 4 μg/ml of ICG concentration is critical threshold with 84 J/cm^2 of light dose. Both of the laser and ICG were non-toxic when they were applied alone.

3.3.4 Dose estimation for ICG-PDT on wild-type strain of *P. aeruginosa*

252 J/cm^2 of light dose was used with 4 different ICG concentrations (50, 100, 125, 150 μg/ml) on *P. aeruginosa* ATCC 27853. This energy dose was not phototoxic when applied alone. Only 1% of decrease in the bacterial load was observed. Similarly none of these ICG concentrations was cytotoxic on this strain when applied alone. 150 μg/ml of ICG caused the highest cytotoxicity and it was only 12% of decrease in the bacterial load and not statistically significant. All the PDT applications caused significant decrease, except the PDT application with 50 μg/ml of ICG. Among them, the combination of 252 J/cm^2 light and 125 μg/ml ICG concentration achieved over 99% killing effect on these gram-negative bacteria (Figure 3.3).

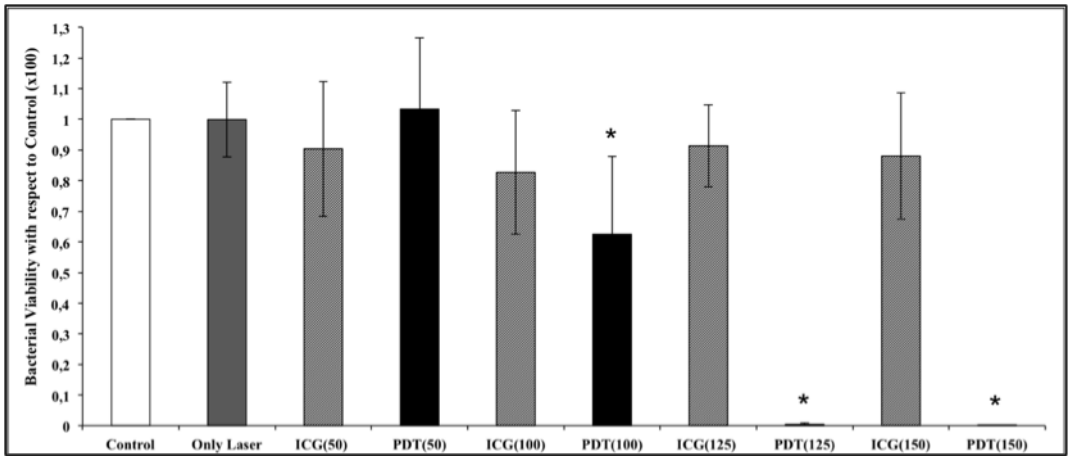

Figure 3.3 Effect of ICG, Laser and PDT applications on wild type *P. aeruginosa* ATCC 27853. Cell viability of *P. aeruginosa* ATCC 27853 after Laser only, ICG only and PDT applications were determined by viable cell count as described above. Bacterial cell count in each experimental group was normalized with the untreated control group (Light dose: 252 J/cm^2 and ICG concentrations: 50, 100, 125, 150 μg/ml). Each column indicates normalized data $\pm$ standard deviation (n>8). $*$ indicates the statistical significance (p<0.05) in comparison to the untreated control group.

3.3.5 ICG-PDT application on resistant strains of *S. aureus* and *P. aeruginosa*

After dose estimation of ICG-PDT on wild type strain *S. aureus* ATCC 25923, the combination of light dose (84 J/cm^2) and ICG concentrations (4 μg/ml and 6 μg/ml) (which were effective on wild type strain) were applied on resistant strain *S. aureus* 1755. It was observed that the applied doses were effective in the photoinactivation of resistant strain. Nearly 100% bacteria were killed after PDT application with these combinations (Figure 3.4).

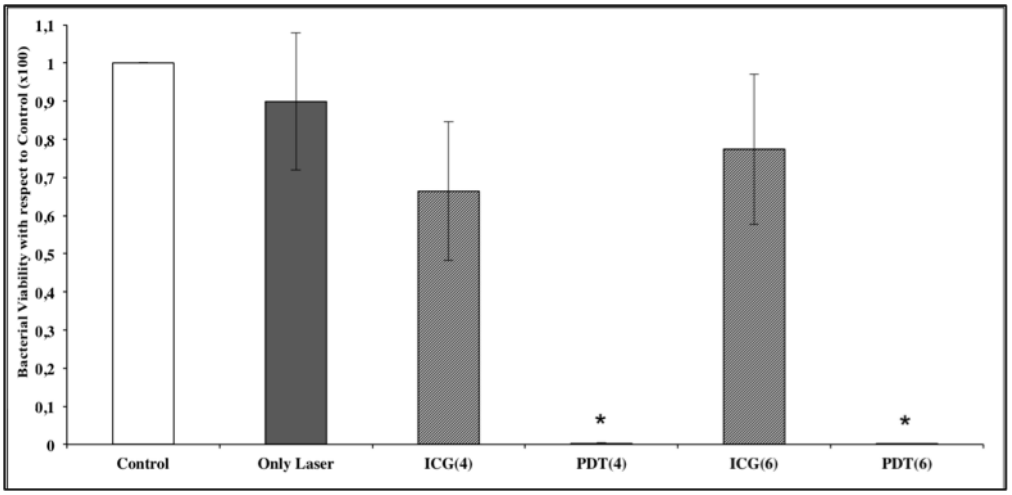

Figure 3.4 Effect of ICG, Laser and PDT applications on resistant strain *S. aureus* 1755. Cell viability of *S. aureus* 1755 after Laser only, ICG only and PDT applications were determined by viable cell count as described above. Bacterial cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2 and ICG concentrations: 4 and 6 μg/ml). Each column indicates normalized data $\pm$ standard deviation (n$>$8). $*$ indicates the statistical significance (p$<$0.05) in comparison to the untreated control group.

Similarly optimum doses (252 J/cm^2 of light dose and 100-125 μg/ml of ICG concentrations) determined for the photoinactivation of wild type *P. aeruginosa* ATCC 27853 were applied on resistant strain *P. aeruginosa* ATCC 19660. These PDT combinations were effective for the photoinactivation of this resistant strain, too. Almost 100% of bacteria had died after the PDT application with 100 μg/ml of ICG whereas for the wild type 60% of the bacteria were alive after the same dose of PDT. PDT with 125 μg/ml of ICG and 252 J/cm^2 of light killed all the bacteria. There were not any viable bacteria counted after this application (Figure 3.5).

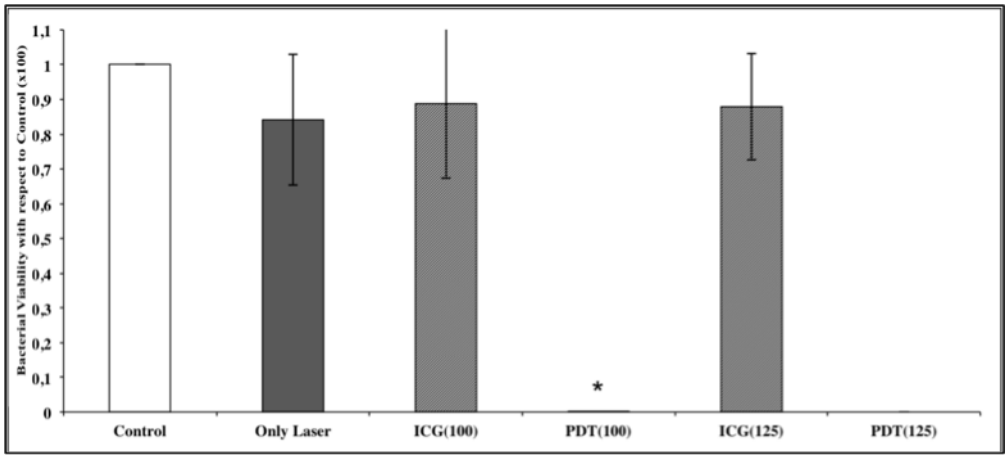

Figure 3.5 Effect of ICG, Laser and PDT applications on resistant strain *P. aeruginosa* ATCC 19660. Cell viability of *P. aeruginosa* ATCC 19660 after Laser only, ICG only and PDT applications were determined by viable cell count as described above. Bacterial cell count in each experimental group was normalized with the untreated control group (Light dose: 252 J/cm^2 and ICG concentrations: 100 and 125 μg/ml). Each column indicates normalized data $\pm$ standard deviation (n$>$8). $*$ indicates the statistical significance (p$<$0.05) in comparison to the untreated control group.

3.4 Discussion

Although the near infrared spectrum is highly advantageous due to its deeper penetration ability to biological tissues, almost all antibacterial PDT studies were performed with visible light. So far the only antibacterial PDT study which reported the lethal photosensitization of ICG on *S. aureus*, *S. pyogenes* and *P. aeruginosa* was by Omar et al [9].

In our study PDT with the combination of ICG and 809-nm light was investigated on wild type and resistant strains of *S. aureus* and *P. aeruginosa*. In the first part, dosimetry study was performed on wild type strains. For gram-positive *S. aureus* same bactericidal effect was achieved with almost 5 fold lower dose of laser (84 J/cm^2) and about 6 times less ICG concentration (4 μg/ml) compared to the study by Omar et al [9]. Similarly, for the gram negative *P. aeruginosa* both the laser dose (252 J/cm^2) and ICG concentration (125 μg/ml) was found to be 2 fold lower. The output power and the bacterial strains are different between our study and the report of Omar et al. These could be the reasons to achieve same bactericidal effect with lower ICG

concentrations. First of all, Omar et al. applied 470 mW as output power to reach 411 J/cm^2 of energy dose [9], whereas 1 W of output power was used in this study. This output power could be strong to kill higher amount of bacteria with lower energy doses. Secondly, bacterial strains of *S. aureus* and *P. aeruginosa* were different between these two studies. The efficiency of PDT seems to be unpredictable and may or may not differ among the different strains of the same species [29, 31, 54]. For instance, Grinholc et al. [53] investigated the effect of protoporphyrin diarginate (PPArg2) on eighty clinical *S. aureus* strains (40 resistant strains and 40 susceptible strains) to reveal the efficiency of photodynamic therapy on different strains. It was concluded that bactericidal effect of PPArg2-PDT was strain dependent with no obvious correlation between photoinactivation and drug resistance.

For the gram negative Pseudomonas species, higher doses of laser energy and ICG compared to *S. aureus* was required for 99% killing efficiency. The difference in ICG concentrations for two different types of bacteria could be explained by the structural differences such as the structure and arrangement of peptidoglycan layers and the presence of outer membrane that is anionic in structure in gram-negative bacteria. The highly anionic nature of outer membrane of Gram negative bacteria will most likely to have a negative impact on the amount of ICG that can either attach or penetrate into the bacterial cell. The preference for wavelengths in visible spectrum that have suitable cationic photosensitizers seems to be the explanation of limited data on near-infrared light and ICG in antibacterial applications.

However our findings on *P. aeruginosa* were still lower than the earlier report and were within the safe working concentrations and four fold less than clinical acne studies [44]. George et al. investigated the bactericidal activity of anionic and cationic photosensitizers on gram positive Enterococcus faecalis and gram negative Actinobacillus actinomycetemcomitans. Cationic methylene blue and rose bengal and anionic ICG were used as photosensitizers with appropriate wavelenghts. It was observed that methylene blue was the most effective photosensitizer among the others. The relatively low efficiency of ICG in this report [51] could easily be explained by insufficient analysis of parameters such as too low energy dose of 3.6 J/cm^2. Among the cationic photo-

sensitizers the efficiency of photoinactivation also differs depending on the structure, amount and distribution of the positive charge. For instance, Banfi et al. investigated the effect of seven different porphrins derivatives in combination with halogen-tungsten lamp with 266 J/cm^2 on *E. coli*, *S. aureus* and *P. aeruginosa* strains *in vitro*. It was reported that cationic derivatives of porphyrin were more effective than the non-ionic derivative of porphyrin and gram positive *S. aureus* was more sensitive to these compounds than the gram negative *E. coli* and *P. aeruginosa* [26].

For the first time the antibacterial effect of ICG-PDT on wild type and drug resistant strains of two different bacterial species were examined comparatively in this study. Keeping the applied energy dose constant for both wild type and resistant strains, it was found that even much lower ICG concentrations had equally effective bactericidal activity on both resistant strains of *S. aureus* and *P. aeruginosa*. Thus it might be speculated that resistant strains of *S. aureus* and *P. aeruginosa* were even more susceptible to ICG-PDT than wild type strains. Although the mechanism is not still understood clearly, even the different strains of the same species of bacteria could respond differently in PDT applications. It was hypothesized that this difference might be due to variation in structures on the outer layers of bacteria which might possibly effect the binding or uptake of the photosensitizer [29, 53]. In parallel with our study, the work by Tseng et al. [27] that investigated 60 different clinical isolates and wild type *P. aeruginosa* had showed that a multi-drug resistant *P. aeruginosa* strain was more susceptible to Toluidine Blue O (TBO)-PDT than non-resistant forms. Similarly, Tang et al. [29] investigated the effect of two cationic photosensitizers, TBO and poly-l-lysine chlorin(e6) (pL-ce6) on wild type and resistant strains of *S. aureus* and *E. coli*. The efficiency of TBO and pL-ce6 was not affected by antibiotic resistivity of these strains.

3.5 Conclusion

In conclusion, this study shows that PDT with non-toxic ICG and low energy doses of 809-nm laser light is an alternative powerful tool to destroy wound-infecting

antibiotic-resistant microorganisms. As a future work, it would be considerably important to investigate reasons of strain-dependent photoinactivation and the effect of output power of light source. It would be valuable to investigate the thermal effect and detect safe thermal area of this wavelength on an animal model during antibacterial PDT. It would lead up wide range of clinical applications. Wound infections could be life threatening for patients with deep burns or diabetic ulcers. Infections easily proceed deep inside these tissue types. PDT with ICG and 809-nm laser is a powerful alternative to treat these infections, because of the penetration capability of this specific wavelength. This method would be also useful for surgical site infections, soft-tissue infections, gastric infections, oral and dental infections [2].

4. ANTIBACTERIAL PDT APPLICATION WITH DIFFERENT OUTPUT POWERS WITHOUT CHANGING LASER ENERGY DOSE ON WILD-TYPE *P. AERUGINOSA* STRAIN: *IN VITRO* STUDY

4.1 Introduction

Mostly preferred photosensitizers in antibacterial photodynamic therapy applications are toluidine blue, methylene blue, ALA derivatives, porphyrin derivatives, chlorin(e6) conjugates, etc. Combination of these photosensitizers with suitable visible light is the commonly used choices in antibacterial photodynamic therapy applications [11, 26, 54, 55, 56, 57]. But visible light has some limitations. Its penetration capability in biological tissue is smaller than the capability of near-infrared light. Near-infrared light is not well absorbed by the main chromophores of the tissue such as melanin and hemoglobin. For example, wavelengths around 800-nm can travel nearly 6 mm through the biological tissue. Thus antibacterial photodynamic therapy with 809-nm light can treat infections in the deeper part of the tissue which cannot be treated by PDT with visible light that reaches only 3 mm in the tissue. Beside it, near-infrared light can be well absorbed by water and this absorption can produce heat in the tissue. Heat production might result in temperature increase, which in turn photothermal interaction may take place. If the output power of the light in this spectrum is high enough, laser light, which irradiates in this spectrum, may cause thermal destruction [25]. In PDT, photochemical mechanism takes places. Pathogens are destroyed by oxygen radicals. Photothermal effect would be the adverse effect for target and neighboring tissue, especially in clinics [5]. Laser power, application duration have to be well-predicted and controlled.

The purpose in this investigation was to determine if PDT with 809-nm diode laser causes any photothermal effect on bacteria to kill them, instead of photochemical

effect. Gram negative bacteria *P. aeruginosa* ATCC 27853 strain was used to see whether higher or lower powers alter the destroying effect of 809-nm light in PDT application.

4.2 Materials and Methods

4.2.1 Bacteria

Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853 was used in this study. This strain was inoculated in tryptic soy broth with shaking at 37°C for overnight. Cells were then harvested by centrifugation and resuspended in phosphate buffered saline (PBS) to approximately 10^8-10^9 CFU/ml.

4.2.2 Photosensitizer

Indocyanine green solutions with a specific concentration were prepared immediately prior to each experiment in PBS and kept at dark. In the ICG and PDT groups, the ICG concentration was 50 μg/ml that is not cytotoxic and/or phototoxic.

4.2.3 Laser Light

In these experiments, computer-controlled 809-nm diode laser was used. It was made in Bogazici University, Biomedical Engineering Institute, Biophotonics Laboratory [52]. In the Laser and PDT groups, the energy dose was 252 J/cm^2. This energy dose was kept constant and achieved with 500, 745, 1000 and 1500 mW output powers by irradiation times 300, 240, 180 and 120 seconds, respectively.

4.2.4 Study Design

The effects of different output powers of 809-nm diode laser during antibacterial PDT application with indocyanine green were studied on *P. aeruginosa* ATCC 27853 *in vitro*. The experimental groups are;

- Untreated control group
- Laser-applied group
- ICG-applied group
- PDT (Laser and ICG applied) group.

50 μl bacterial suspensions were transferred into the wells of 96-well plate. In the ICG and PDT groups, 50 μl of ICG solution in PBS was added to each well and mixed well with bacteria. After addition of ICG, the wells were kept at dark and incubated for 15 minutes. To apply the same conditions to all groups, the bacterial suspension in the wells was mixed with equal volume of PBS (50 μl) in the Laser and Control groups. Then bacterial suspensions were irradiated by 809-nm diode laser in PDT and Laser groups. Following each application, viable cell count was determined by serial dilution method.

4.3 Results

In this study, 4 different output powers were applied to observe whether they have any photothermal effects on the cell viability. By changing exposure duration, energy dose was kept constant at 84 J/cm^2. In the laser-applied groups, changing output power did not alter bacterial viability. Determined cell count in these groups was nearly same as the cell count in untreated control group. We did not observe any statistically significant increase or decrease in cell viability after laser application (Figure 4.1).

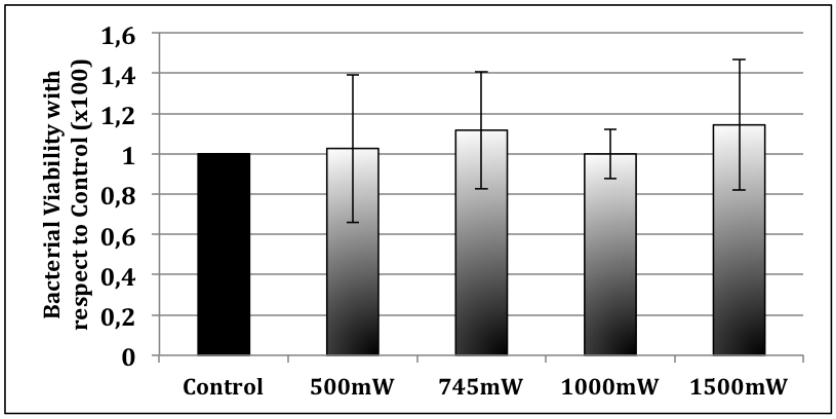

Figure 4.1 Effect of Laser application with different output powers without changing energy dose on wild type *P. aeruginosa* ATCC 27853. Cell viability of *P. aeruginosa* ATCC 27853 after Laser-only applications were determined by viable cell count as described above. Bacterial cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2 and Output powers: 500, 745, 1000, and 1500 mW) Each column indicates normalized data $\pm$ standard deviation ($n>8$). $*$ indicates the statistical significance ($p<0.05$) in comparison to the untreated control group.

Later these 4 different powers were examined in PDT applications. Energy dose was kept constant at 84 J/cm^2 by changing exposure duration. ICG concentration was 50 μg/ml. In our previous studies we observed that this concentration was not cytotoxic when applied alone and not phototoxic when applied with laser as shown in the Figure 4.2 There was a small decrease in bacterial cell viability after 15-minute incubation with ICG but it was not statistically significant. Bacterial proliferation was observed with 500 mW of output power in the PDT group. This was an increase more than 20% and statistically significant. In the other PDT groups, bacterial viability was nearly the same as in the untreated control groups. There were slight decrease or increase in cell viability but these changes were not statistically significant (Figure 4.2).

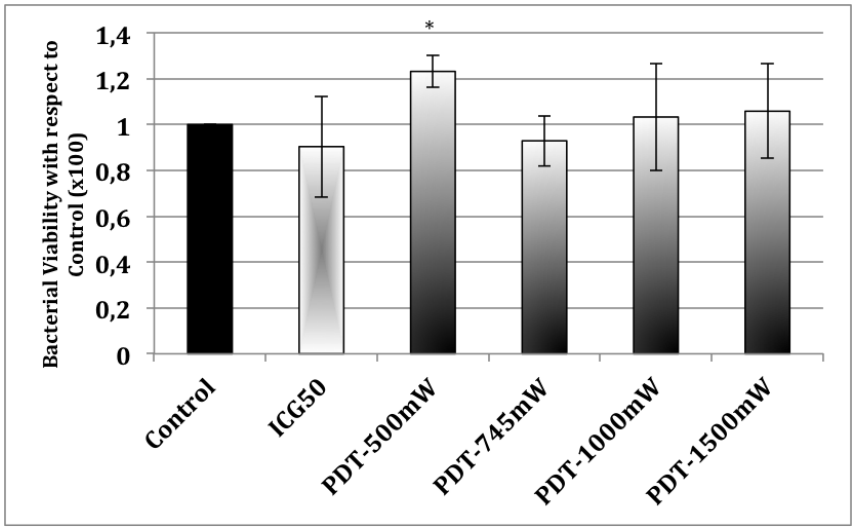

Figure 4.2 Effect of ICG, and PDT applications with different output powers on wild type *P. aeruginosa* ATCC 27853. Cell viability of *P. aeruginosa* ATCC 27853 after ICG-only and PDT applications were determined by viable cell count as described above. Bacterial cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2, Output powers: 500, 745, 1000, and 1500 mW and ICG concentration: 50 μg/ml). Each column indicates normalized data $\pm$ standard deviation (n$>$8). $*$ indicates the statistical significance (p$<$0.05) in comparison to the untreated control group.

4.4 Discussion

In previous experiments, our group found out the optimized parameters (laser dose and photosensitizer concentration) of antibacterial photodynamic therapy with ICG to destroy gram negative *P. aeruginosa* strains [58]. Efficient 99% killing effect of PDT was achieved with lower laser dose and ICG concentration when compared to earlier reports [9].

The major difference between our study and the earlier report is the output power. Thus we tried to find out whether there is a thermal destruction due to 809-nm wavelength. These might be the reason to achieve same bactericidal effect with lower ICG concentrations in our studies. First of all, Omar et al. applied 470 mW as output power to reach 411 J/cm^2 of energy dose [9], whereas 1 W of output power was used to reach 252 J/cm^2 of energy dose in our previous study [58]. It was thought that 1 Watt of 809-nm light could be strong to kill higher amount of bacteria with lower energy doses.

Figure 4.1 showed that there was not any thermal destruction when laser applied alone. The data obtained from laser groups were very close to the data of untreated control group. Then this hypothesis was tested in PDT groups. In this part of the experiments, 50 μg/ml of ICG was used to eliminate the effect of photosensitizer. We demonstrated that this concentration was not enough to produce effective oxygen radicals to destroy bacteria when applied together with laser [58]. In the presence of ICG, 809-nm light is absorbed more intensely. Thus it may lead to more heat production and then temperature increase depending on the high absorption capability of indocyanine green. This photothermal effect was not observed in the PDT groups, too. There was not any decrease in the bacterial cell viability depending on the thermal destruction. Beside it, there was a significant increase in the PDT group with 500 mW output power which means that bacterial cells have proliferated after PDT application.

These experiments clarified that changing the output power in the range of 500 mW to 1500 mW did not affect the mechanism of photodynamic therapy with near-infrared laser. In this output power range, the only mechanism to destroy bacteria was the photochemical mechanism via oxygen radical formation.

4.5 Conclusion

Wavelengths in near-infrared spectrum are more powerful to treat deeper infection than wavelengths in visible spectrum, depending on their penetration capability in biological tissue. Actually, their mechanism of action with tissue can be photothermal and these can cause adverse effect during photodynamic therapy. But this study shows that output powers around 1 Watt did not cause any death of bacteria depending on thermal destruction during PDT applications *in vitro*.

5. BIOSTIMULATION RISK DURING ANTIBACTERIAL PDT WITH NEAR-INFRARED LASER LIGHT ON WILD-TYPE *P. AERUGINOSA* STRAIN

5.1 Introduction

Photodynamic therapy (PDT) combines the use of light sensitive drug (photosensitizer) with a suitable wavelength to activate the photosensitizer. Activated photosensitizers cause lethal damage to the target [7, 18, 21, 57]. The cytotoxicity of the photosensitizer depends on the light and oxygen molecules, which are available in the environment. The light absorbed by photosensitizer results in the transfer of energy to molecular oxygen yielding highly reactive oxygen radicals [18, 21]. Cellular responses to oxygen radicals 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 [59, 60, 61, 62].

We investigated the antibacterial effect of indocyanine green (ICG) with 809-diode laser *in vitro* which was explained in Chapter 2. Most effective ICG concentrations and energy dose were determined on wild type strains of *S. aureus* and *P. aeruginosa*. Then they were applied on resistant strains of these bacteria. PDT with ICG was totally efficient to kill all of these bacterial strains. Neither light and nor ICG alone caused any lethal effect on any of the strains. More than 95% killing effect was achieved with 4 μg/ml of ICG concentrations with 84 J/cm^2 of light dose on resistant strain of *S. aureus*. Optimum parameters for 99% killing of resistant strain of *P. aeruginosa* were 100 μg/ml ICG and 252 J/cm2 of light dose. Temperature measurements also showed that there was no photothermal effect in that bactericidal effect. Thus, it was concluded that 809-nm laser and ICG combination yielded a Photodynamic effect without any temperature increase [58].

However, during the predosimetry studies, we observed some unexpected results that aroused the question of 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 fails but also proliferation of the pathogens could be observed. This might be the result of biostimulative effect of laser or laser-photosensitizer combination.

The main purpose of this study was to investigate the risk of biostimulation during ICG-PDT application. Specifically, PDT dosimetry (light dose and concentration of photosensitizer) on *P. aeruginosa* was studied and searched for the safe regions or ranges of antibacterial laser applications.

5.2 Materials and Methods

5.2.1 Bacteria

Gram-negative bacteria *P. aeruginosa* ATCC 27853 (Gazi University, Ankara, Turkey) was used in this *in vitro* study. Single colony was inoculated into tyrptic soy broth and incubated overnight at 37°C. Bacterial cells were harvested by centrifugation (3000 rpm for 10 minutes at 4°C) and the pellet was resuspended in phosphate buffered saline (PBS) to approximately 10^8-10^9 colony-forming unit/ml (CFU/ml).

5.2.2 Photosensitizer and Laser

ICG (Pulsion Medical System AG, Munich, Germany) solution was prepared in PBS before each experiment and kept in the dark. ICG concentrations were between 20-250 μg/mL. Absorption and fluorescence maxima of ICG are around 800-nm. Therefore, 809-nm diode laser was used for PDT experiments. This laser was built in Bogazici University, Biomedical Engineering Institute, Biophotonics Laboratory [52]. 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, CA, USA). The energy doses (84, 168 and 252 J/cm^2) were applied by increasing the exposure duration (60, 120 and 180 seconds respectively).

5.2.3 Study Design

Experimental groups were Control group, Laser groups, ICG groups and PDT groups.

- **Laser Groups:** L84, L168, L252 denoting the Laser irradiation doses (J/cm2).
- **ICG Groups:** ICG20, ICG50, ICG100, ICG125, ICG150, ICG200, ICG250 denoting the photosensitizer concentrations (μg/mL).
- **PDT Groups:** PDT84/20, PDT84/50, PDT84/100, PDT84/125, PDT84/150, PDT84/200, PDT84/250, PDT168/20, PDT168/50, PDT168/100, PDT168/125, PDT168/150, PDT168/200, PDT252/20, PDT252/50, PDT252/100, PDT252/125, PDT252/150 denoting PDT laser doses and photosensitizer concentrations as PDT (J/cm^2)/(μg/mL).
- **Control Group**

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 dark for 15 minutes. 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 sample were plated on tyrptic soy agar. After 24-hour incubation in dark, 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.

5.2.4 Statistical Analysis

In each experiment, some of the wells were used for experimental groups and others were assigned to control samples. In order to keep the conditions constant, viable cell counts determined after serial dilution method were normalized by taking the ratio with corresponding control groups on each 96-well plate. Normalized data were analyzed for statistical significance with one-way ANOVA and two-tailed Student?s t-test. The significance level was $p < 0.05$.

5.3 Results

The main purpose of this study was to investigate the risk of proliferation of bacterial cells after the applications of PDT with low-dose of laser and photosensitizer. Initially the effect of 84 J/cm^2 of energy dose was investigated with 7 different ICG concentrations (20, 50, 100, 125, 150, 20, 250 μg/mL). 84 J/cm^2 energy dose caused around 10% increase in cell number when applied alone. When ICG applied alone, slight decrease in cell number was observed between the ranges of 50-200 μg/mL. Maximum decrease was with 100 μg/mL of ICG and it was nearly 20%. Statistically significant amount of cell proliferation was observed when ICG concentrations were 20 μg/mL and 250 μg/mL and it was around 30% (Figure 5.1).

In PDT groups, 84 J/cm^2 of light with 20, 50, 100, 125 and 150 μg/mL of ICG resulted in statistically significant amount cell proliferation (61%, 18%, 32%, 40% and 38% respectively). Increasing ICG concentration further to 200 and 250 μg/mL caused only 28% of cell death in these PDT groups and it was not desired bactericidal effect of PDT (Figure 5.1).

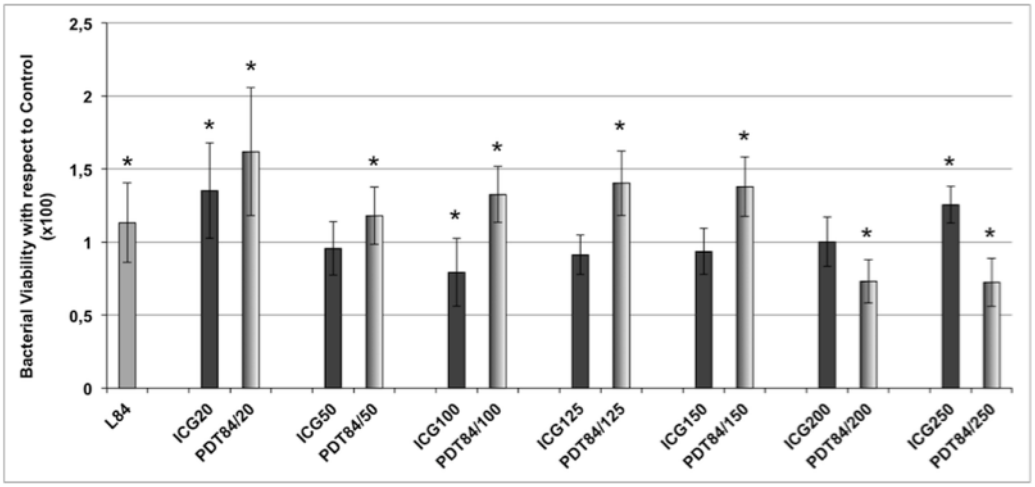

Figure 5.1 Bacterial viability percentage after Laser, ICG and PDT applications on *P. aeruginosa* ATCC 27853. Viability of *P. aeruginosa* ATCC 27853 was determined after Laser only, ICG only and PDT applications. Cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150, 200, 250 μg/mL). Each column indicates normalized data $\pm$ standard deviation (n$>$8). $*$ indicates the statistical significance ($p<0.05$) in comparison to the untreated control group.

When 168 J/cm^2 of energy dose was applied alone, a decrease around 15% was observed. Then this energy dose was applied with the same ICG concentrations. 168 J/cm^2 of energy dose with 20 μg/mL of ICG caused around 12% increase in viable cell count. The cell number after the application with 168 J/cm^2 of energy dose and 50 μg/mL of ICG was quite same as the cell number in untreated control group. By increasing the ICG concentration, photoinactivation of bacteria was observed starting from the concentration of 100 μg/mL (Figure 5.2). To the end of this ICG concentration range, the desired PDT effect was achieved by killing 99% bacterial load.

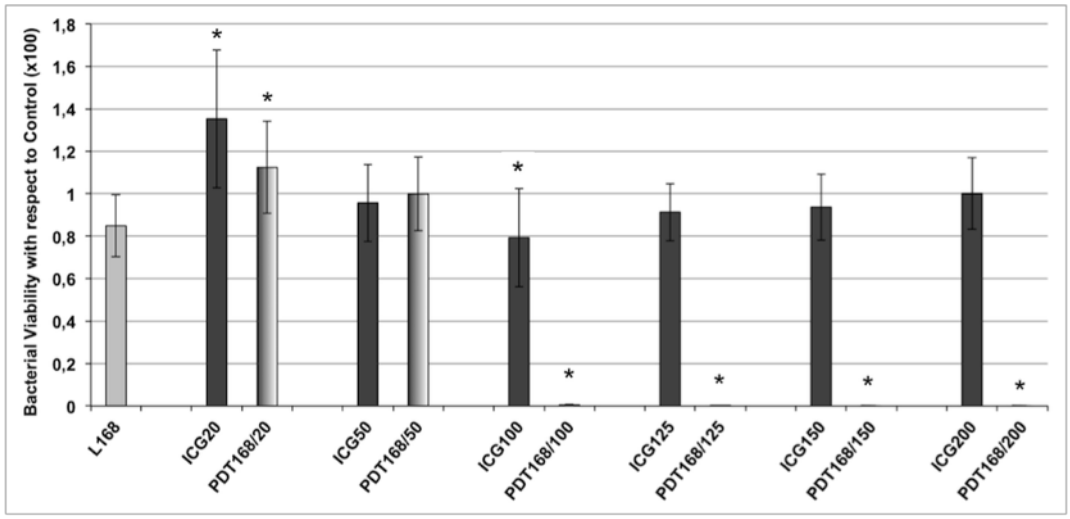

Figure 5.2 Bacterial viability percentage after Laser-only, ICG-only and PDT applications on *P. aeruginosa* ATCC 27853. Viability of *P. aeruginosa* ATCC 27853 was determined after Laser only, ICG only and PDT applications. Cell count in each experimental group was normalized with the untreated control group (Light dose: 168 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150, 200 μg/mL). Each column indicates normalized data $\pm$ standard deviation (n$>$8). $*$ indicates the statistical significance (p$<$0.05) in comparison to the untreated control group.

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 viable cell count when applied alone. With this energy dose, 20 μg/mL of ICG had a proliferative effect on bacterial cell count. It was around 40%. 50 μg/mL of ICG had no effect on viable bacteria; it was same as untreated control group. Starting from 100 μg/mL of ICG, bactericidal effect of PDT was observed in these groups. 150 μg/mL of ICG was efficient enough to kill 99% bacterial load (Figure 5.3).

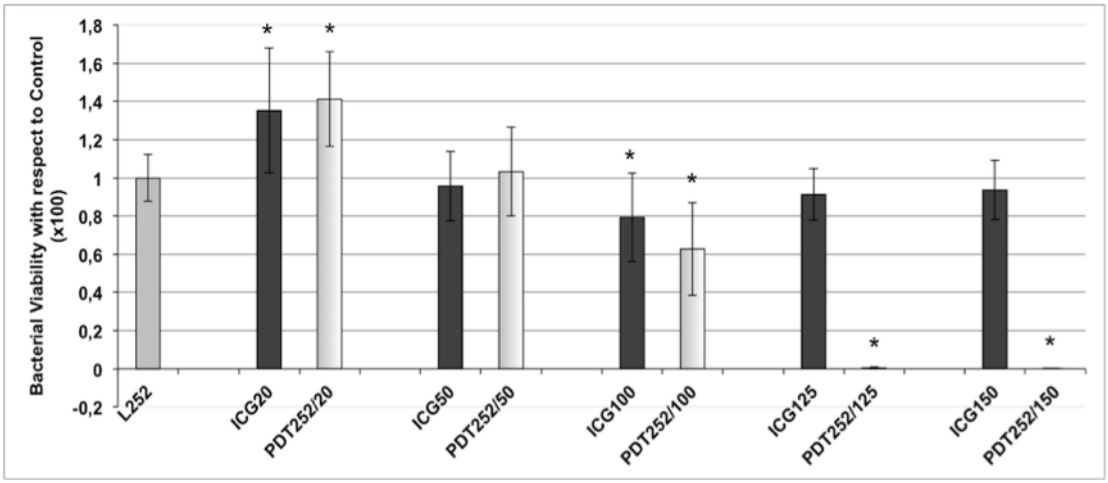

Figure 5.3 Bacterial viability percentage after Laser, ICG and PDT applications on *P. aeruginosa* ATCC 27853. Viability of *P. aeruginosa* ATCC 27853 was determined after Laser-only, ICG-only and PDT applications. Cell count in each experimental group was normalized with the untreated control group (Light dose: 252 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150 μg/mL). Each column indicates normalized data $\pm$ standard deviation (n$>$8). $*$ indicates the statistical significance ($p<0.05$) in comparison to the untreated control group.

Experiments showed that combination of laser with photosensitizer could be resulted in photoinactivation if the doses of irradiation and concentration of ICG were selected in a specific range; otherwise PDT application could be resulted in the proliferation of bacteria. Thus both parameters were plotted in order to visualize the ranges of the dosimetry for safe bactericidal effect and biostimulative effect (Figure 5.4).

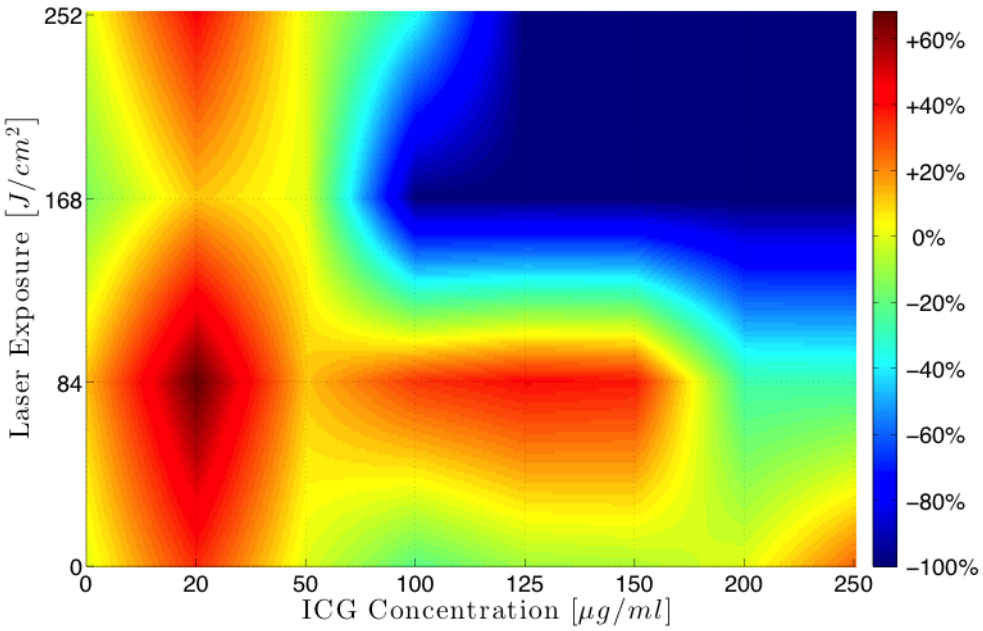

Figure 5.4 Pseudo colors showed the percentage increase or decrease of the bacteria population, red indicates the proliferative effect and blue shows the antibacterial effect.

5.4 Discussion

One of the proposed mechanisms of PDT with indocyanine green and wavelength around 800-nm light is photochemical mechanism, which is photooxidative. Szeimies and his colleagues investigated photooxidative killing effect of indocyanine green 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. While using histidine and sodium azide, which are quenchers of singlet oxygen, killing efficiency of PDT decreased. While using extra singlet oxygen source, photooxidative effect on cancer cells increased [35].

The efficacy of PDT mainly depends on light and drug dose as well as the time interval between drug addition and light application. When sufficient incubation time is allowed, reactive oxygen species are produced as a result of interaction between light and drug. These reactive oxygen species are responsible for the cell death. The

amount of oxygen radicals generated depends on the concentration of drug, light dose and the amount of molecular oxygen that are available in the environment [7, 18, 21, 57]. When drug and light dose are kept low, the amount of oxygen radicals will be low too. Therefore their destroying effect is expected to be low [21]. However lower concentration of reactive oxygen species can stimulate different biochemical pathways in the cells causing cell survival and proliferation [63, 64, 65, 66, 67, 68, 69, 70]. The two distinct effects of laser applications were studied in independent tracts in literature, i.e. proliferation effect for the cells [71] or inhibitory/killing effect for unwanted cells leading to cancer tissue [36, 37, 72]. This is the first time; those of the 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. After increasing ICG concentration, expected killing effect was observed but it was not enough to kill 99% bacteria. 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 greater than 168 J/cm^2 laser irradiation and higher than 100 μg/mL ICG should be combined in order to yield an efficient photoinactivation of *P. aeruginosa* ATCC27853. Combination of ICG less then 100 μg/mL and laser irradiation less then 168 J/cm^2 results a proliferative effect on bacteria; we can call this situation as biostimulation. This biostimulative effect turned to bactericidal after a neutral region of doses.

The safe region for bactericidal usage was found clearly (Figure 5.4) but some irregularities were also observed for only-ICG groups. The lowest (20 μg/mL) and the highest ICG concentrations (250 μg/mL) used in this study increased cell viability when applied alone. Sato et al. observed similar results in their study [73]. Incubation of cultured Muller cells with ICG at low concentrations promoted cell viability. It is supposed that immediate cellular responses to lower concentrations are to activate cell

survival pathways, similar to the conditions under oxidative stress. However, a higher concentration of ICG alone (250 μg/mL) resulted a biostimulative effect unexpectedly. Further investigations focused on ICG related cellular stress are needed in order to reach an explanation.

5.5 Conclusion

When light and photosensitizer dose are not optimized properly and kept at lower doses in PDT, reactive oxygen species stimulate different cellular pathways and cause cell proliferation in the end. Therefore optimum dosimetry in PDT possesses great importance in the treatment of wounds infected by antibiotic-resistant bacteria.

6. ANTIBACTERIAL PDT APPLICATION ON INFECTED WOUND MODEL BY ICG AND 808-NM DIODE LASER: *IN VIVO* STUDY

6.1 Introduction

Many strains of *Staphylococcus aureus* and *P. aeruginosa* are the main cause of nosocomial or superficial skin infections. They are highly pathogens and easily colonize in biological tissue. It is difficult to treat antibiotic-resistant strains by common antibiotics. Bacterial biofilm formation makes the treatment with conventional methods more difficult, even impossible [6, 9, 14, 74, 75, 76, 77, 78]. Inefficacy of antibiotics and other treatment techniques lead researchers to find out, to assess, and to investigate more effective and successful modalities to solve this worldwide health problem [58]. Photodynamic therapy (PDT) is thought to be life-saving solution at this point.

Suitable wavelengths for PDT mechanism are in the visible and near-infrared spectrum. The most preferred wavelengths are in the range of 632,5-650 nm. Photosensitizers that highly absorb visible light are advantageous for antibacterial photodynamic action because of their cationic nature, which is attractive to anionic structure of bacterial cell [18, 22, 79]. Characteristics of the wavelengths in near-infrared spectrum are more advantageous for the applications on biological tissues. Wavelengths around 780-810 nm can penetrate deep inside the tissue, nearly 6 mm which is nearly twice as the depth as which visible light travel through the tissue [25]. This feature may provide an opportunity for eliminating deeper infections. Appropriate photosensitizer for near-infrared spectrum is indocyanine green whose anionic nature decreases its availability as an antibacterial agent [58]. There are numerous successful tumor studies performed on animal model using ICG and near-infrared light together, but there is not any antibacterial *in vivo* study using these modalities because of the anionic nature of ICG.

In this study, we aimed to show efficiency of ICG-808 nm combination in photodynamic therapy applications on abrasion wound model infected with resistant-strain of *S. aureus* and *P. aeruginosa*. Its effect was evaluated by determining bacterial burden on infected wounds after each application, examining morphologies and histological sections of the wounds. Finally temperature change was measured to determine the heating effect on the target tissue.

6.2 Materials and Methods

6.2.1 Bacteria

Multidrug resistant strains of *Staphylococcus aureus* (1755) and *P. aeruginosa* (ATCC 19660) were used in this study to infect the wounds. *Staphylococcus aureus* 1755 was a clinical isolate obtained from Microbiology department of Gazi University, Ankara, Turkey. *P. aeruginosa* ATCC 19660 was purchased from ATCC, USA.

These bacteria were subcultured on tryptic soy agar, then single colony was inoculated in tryptic soy broth overnight at 37°C. Bacterial suspension was then centrifuged, supernatant was discarded and the pellet was dissolved in phosphate-buffered saline (PBS). This solution was used to infect the wounds on animals.

6.2.2 Photosensitizer and Light Source

ICG (Pulsion Medical Systems AG, Munich, Germany) solution was prepared freshly in PBS before each experiment and kept in dark to protect it from photobleaching. All the experiments in which ICG was used were also performed at dark.

808-nm diode laser was used as a light source (Figure 6.1). It is tunable and continuous-mode laser with a maximum output power of 2-W. Laser light was delivered to the target tissue with a 1000-μm optical fiber, which was coupled to the original

fiber of the laser. To illuminate an area of 1-cm^2, a collimator was used attached to the end of 1000-μm optical fiber. The distance between the tip of optical fiber and the target tissue was fixed and the power of the laser was controlled before each experiment with a power meter (Newport 1918-C, CA, USA).

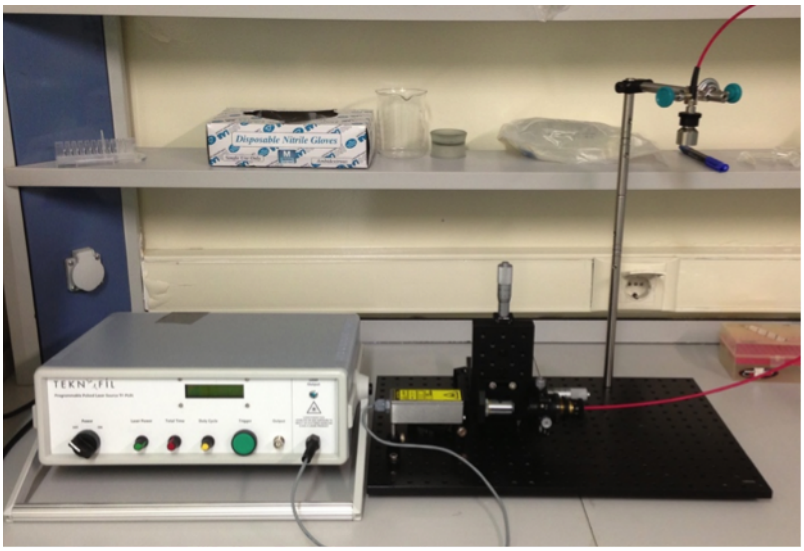

Figure 6.1 808-nm diode, which is tunable, and continuous-mode laser with a maximum output power of 2-W and laser setup for beam transfer between fibers.

6.2.3 Animals

Randomly selected wistar albino female rats, 2-3 months old, weighing 170-220 g were used in this study. They were obtained from Vivarium, Center for Life Sciences and Technologies Research at Bogazici University. All experiments were approved by Institutional Ethics Committee for the Local Use of Animals in Experiments of Bogazici University. Animals were anesthetized by intraperitoneal (i.p.) injection of ketamine and xylazine mixture (90 mg/kg ketamine, 10 mg/kg xylazine) before wound creation and laser application.

6.2.4 Wound Models (Excisional and Abrasion Wounds)

Excisional wounds: Dorsal hair of the animals was shaved by an electronic razor, and then the skin was cleansed by 70% (v/v) alcohol. To open one full-thickness excisional wound model, subcutaneous skin was lifted by the help of sterile forceps and approximately 8 mm circular area was cut through the panniculus carnosus by sterile scissors. Nearly 50 mm2 wound was opened through subcutaneous areolar tissue (Figure 6.2). Then 50 μl of bacterial solution was added into the wound by the help of a pipette. There was 30 minutes of waiting period after adding bacterial suspension for the diffusion of bacteria deep inside the tissue.

Abrasion wounds: Dorsal hair of the animals was shaved by an electronic razor, and then the skin was cleansed by 70% (v/v) alcohol. To create abrasion wounds, 21-gauge needles were used to scratch an area of approximately 1 cm^2 on the upper layer of the epidermis (Figure 6.2). After creating the wounds, 50 μl of bacterial suspension dissolved in PBS was added to the scratched area of the wound by help of the tip of a pipette. There was 30 minutes of waiting period before any following application for the inoculation of bacteria in the wound.

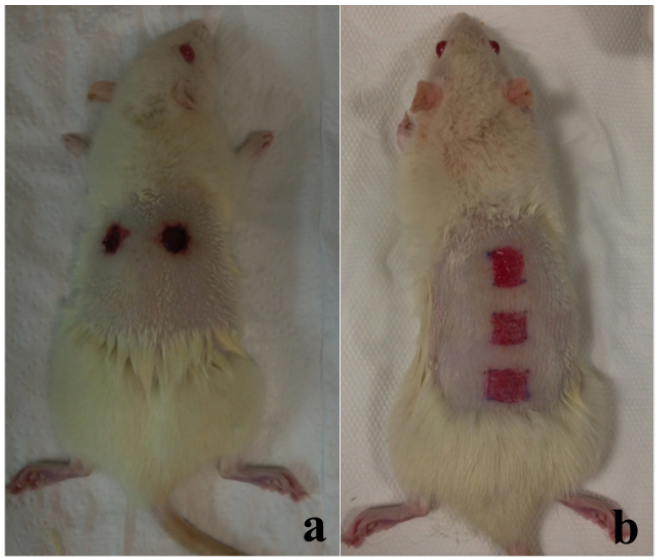

Figure 6.2 a) Excisional wound model, b) Abrasion wound model

6.2.5 Determination of thermally safe area during PDT application with 808-nm diode laser and ICG

To analyze the thermal effect of laser irradiation, temperature of the wounds were measured by a 20-μm K-type thermocouple which has a response time of 0,1 second and measures changes of 0,1°C (Figure 6.3). The temperature of the wound was measured firstly before laser irradiation to determine temperature increase after irradiation. Then temperature increase was measured in the presence and absence of ICG immediately after illumination.

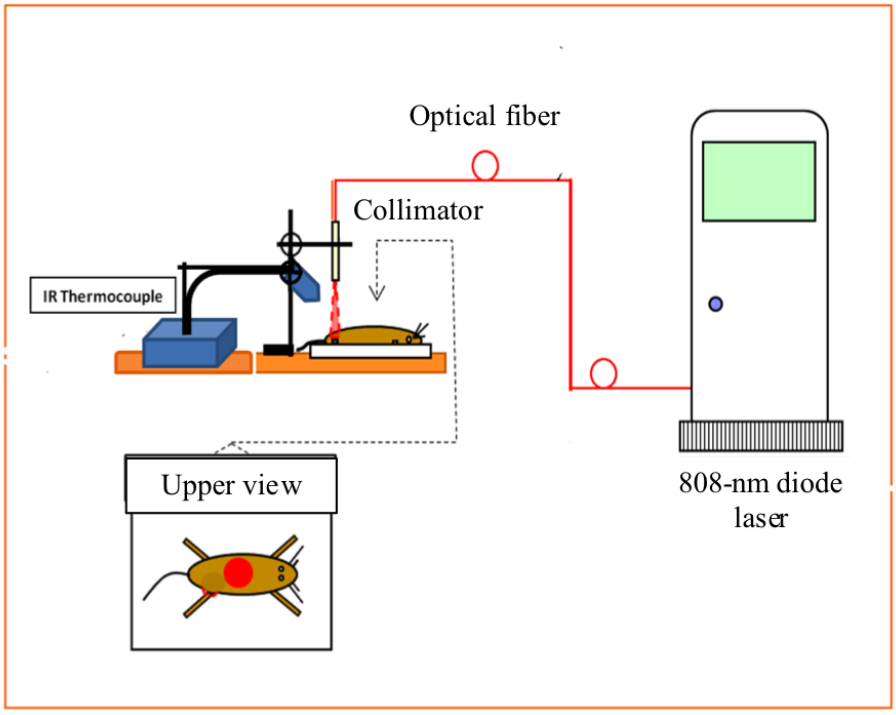

Figure 6.3 Schematic representation of laser setup and IR thermocouple system.

6.2.6 ICG-IR Laser PDT application on infected excisional wounds

After incubation of bacteria in the wound, 50 μl of ICG solution was added into the wound and incubated for 30 minutes for the diffusion of ICG. Approximately 1 cm^2 area of skin, which included the wound, was irradiated by laser. At the end of treatments, wound area was excised aseptically and transferred in 5 mL of PBS.

The weights of the samples were calculated, and then the tissue in buffer solution was compressed to release the bacteria within the tissue by a sterile pestle. Tissue samples treated with photosensitizer were kept at dark during processing to prevent photobleaching of ICG. The aliquots were serially diluted in PBS by 1/10 dilution factor and then were plated onto tryptic soy agar and incubated overnight at 37°C. Colonies counted on these plates were then multiplied by dilution factor to calculate the amount of bacteria within the corresponding tissue sample. Then colony-forming units (CFU) per gram was calculated for each wound.

6.2.7 ICG-IR Laser PDT application on infected abrasion wounds

ICG solution was added to the wound after inoculation of bacteria. Immediately after the addition of ICG solution, irradiation of the wound by laser was started. First, 10 μl of ICG solution was added and additional 10 μl of ICG was added to the wound at 3-minute intervals until the total volume of 50 μl (5x10 μl) was completed during laser application. Laser irradiation lasted for 15 minutes in each application. Output power was 500 mWatt and therefore laser energy dose transferred to the wound was 450 J/cm^2 (Figure 6.4).

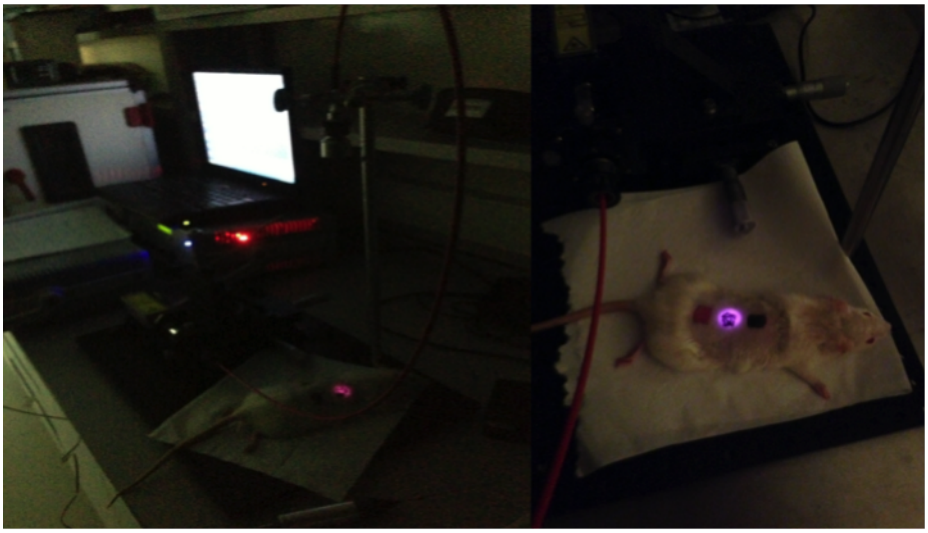

Figure 6.4 Laser application on infected abrasion wound model at dark.

Beside PDT applied wounds, an additional wound was created as a control wound on each animal. Neither ICG nor laser was applied to these control wounds. To compare bacterial cell viability, only laser or only ICG was applied to some wounds. In laser group, wounds were irradiated with 450 J/cm^2 of laser energy without any ICG. In ICG group, specific ICG concentrations used in PDT groups were added to the wounds without any laser irradiation. In table 6.1 and 6.2, applied parameters on infected wounds were shown.

Table 6.1
Output powers and ICG concentrations that were used on wounds infected with *S. aureus 1755.*

Output Powers (Watt)	Exposure duration (Seconds)	ICG concentration (µg/ml)
0,9	75	4
		6
		10
		40
		50
		100
		250
		400
		500
0,5	300	500
0,5	600	1000
0,3	1800	1000
		1500
0,2	1800	4
		10
		25
		50
		75
0,2	900	1000

Table 6.2
Output powers and ICG concentrations that were used on wounds infected with *P. aeruginosa* ATCC 19660

Output Powers (Watt)	Exposure duration (seconds)	ICG concentration (µg/ml)
0,9	210	150
		500
		750
		1000
		1500
0,5	900	1000
0,3	1800	1000
0,2	1500	100
		150
		250

Following these applications, wounds were removed from the animals by sterile scissors and forceps. Tissue samples were cut at the boundaries of the wounds and these samples were put in 5 ml of PBS. The weights of the samples were calculated, and then they were compressed in buffer solution to release viable bacteria from the tissue by using a sterile pestle. Tissue samples treated with photosensitizer were kept at dark during processing to prevent photobleaching of ICG. Viable bacteria in these solutions were calculated using serial dilution method by diluting 1/10 fold. Diluted samples were placed on tryptic soy agar plates and incubated overnight at 37°C (Figure 6.5). Colonies counted on these plates were then multiplied by dilution factor to calculate the amount of bacteria within the corresponding tissue sample. Then colony-forming units (CFU) per gram was calculated for each wound.

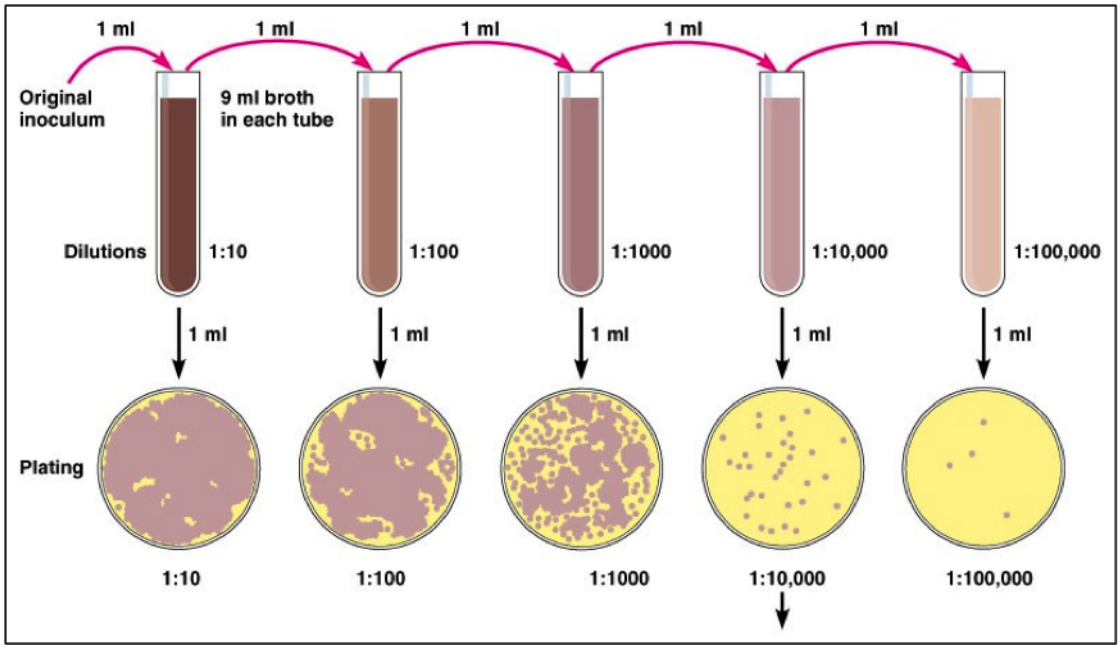

Figure 6.5 Schematic diagram of serial dilution method.

6.2.8 Wound healing and histological analysis

In order to observe wound healing period, 2-day, 4-day, 7-day and 11-day groups were formed from 4 animals with a single wound on each of them in each group and optimum combination of ICG concentration and energy dose to destroy bacteria effectively defined after PDT applications were used to treat infected wound on each animal and then these animals were followed and examined for 2, 4, 7 and 11 days. On treatment day, the initial size of the wounds was precisely measured by vernier calipers. Following each day, the size of the wounds was measured again until the day specified for each group arrived, and then the animals were sacrificed to remove wounds for histological analysis. For ethical consideration, animals with untreated wounds were not included to these analyses, not to keep animals alive without any treatment for a time period. For histological analysis of the wounds before and immediately after PDT treatment, 2 groups were formed from 3 animals with 2 wounds on each as control groups to compare with treated ones and these animals were sacrificed following the applications.

Removed tissue samples were fixed in 10% PBS-formalin solution for 2-3 days. After fixation, samples were placed in cassettes and processed in Tissue Processor (Leica TP 1020). Dehydration procedure is explained below in Table 6.3.

Table 6.3
Dehydration procedure.

Alcohol	70%	1 hour
Alcohol	80%	1 hour
Alcohol	80%	1 hour
Alcohol	96%	1 hour
Alcohol	96%	1 hour
Alcohol	100%	1 hour
Alcohol	100%	1 hour
Alcohol	100%	1 hour
Alcohol	100%	1 hour
Paraffine (60°C)		1,5 hour
Paraffine (60°C)		1,5 hour

These samples were embedded in paraffin blocks and sectioned to 6-μm thickness by microtome (Leica RM 2255). These sections were placed on slides and stained with hematoxylin-eosin. Staining procedure is shown and summarized in Figure 6.6. Stained slides were assessed under a light microscope (Nikon Eclipse 80i, Japan) to observe the epithel lining, re-epithelization, inflammation and collagen formation.

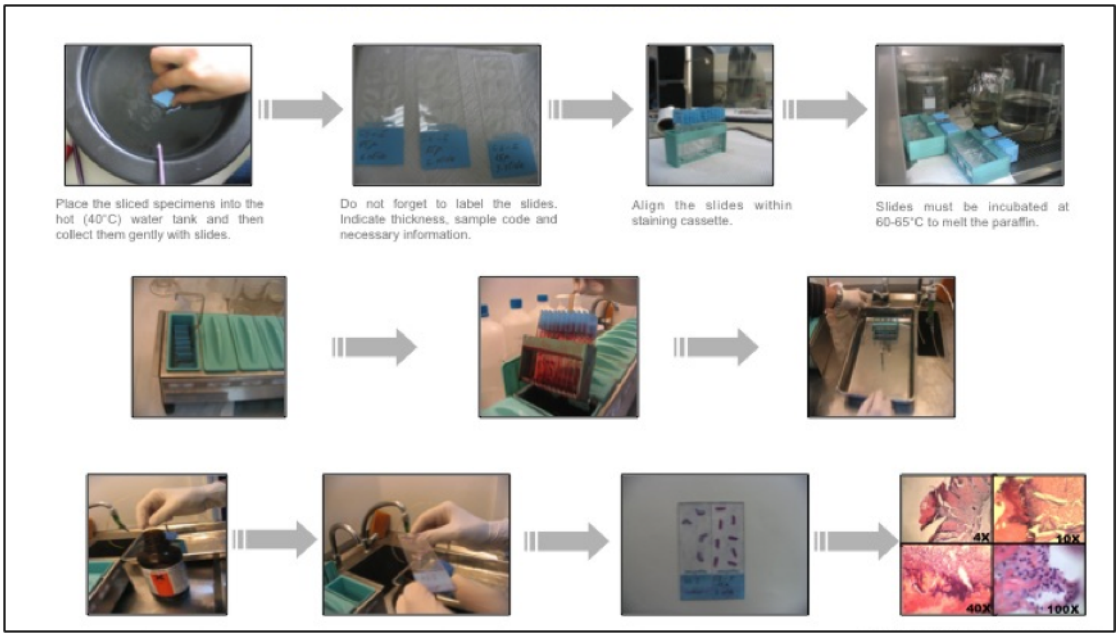

Figure 6.6 Hematoxylene-Eosin staining procedure.

6.2.9 Statistical analysis

All the viable bacterial cell count data obtained after any application were normalized by dividing it with its corresponding data of control wound. These normalized data were analyzed with one-way ANOVA and then two-tailed Student?s t-test for statistical significance. p values those were lower than 0.05 were considered as significantly different.

6.3 Results

6.3.1 Optical setup configuration of 808-nm diode laser

A proper optical setup for 808-nm diode laser was configured not to damage the original fiber of the laser and neighboring tissue of the wound thermally, to illuminate a definite area homogenously.

In diode lasers, light gets out of the fiber divergingly and has a Gaussian profile. The aim was to use homogenous part of this Gaussian profile to illuminate the wound, covering it wholly. Beam expander and iris diaphragm was used to provide a homogenous illumination area. An optical fiber with a diameter of 1000 μm was coupled to this setup to prevent damaging original fiber and a collimator was attached to the tip of this fiber to prevent fluctuations in spot size (Figure 6.7).

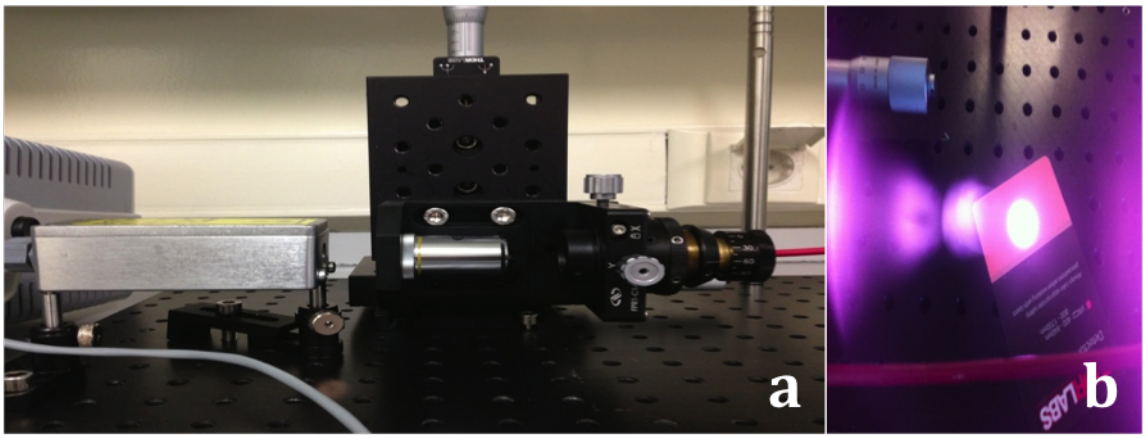

Figure 6.7 a) Optical setup for laser beam transfer between the fiber of the laser and another laser fiber which was 1000 μm. a) View of the laser beam on reader card.

6.3.2 Temperature Measurements

Temperature change was measured by 20-μm K-type thermocouple, which has a response time of 0,1 second and measures changes of 0,1 °C. ICG is a high absorbant at NIR region. Therefore it was supposed to observe higher temperature increase in the presence of ICG after laser application. Temperature of the tissue was 22,54±0,29°C before laser application. When 300 mW of output power was applied for 30 minutes, it caused 1°C of temperature change. In presence of ICG, this application resulted in 6,41°C of temperature change. When 500 mW of output power was applied for 15 minutes, 450 J/cm^2 of energy was delivered to the tissue and caused only 1,32°C temperature change. In the presence of ICG, temperature change was 7,68°C after same amount of illumination. ICG caused more than 6°C change. During laser illumination, maximum temperature reached was 39,53°C. It was still below critical point of 45°C.

When 900 mWatt was applied for 75 seconds, measured temperature change was 15°C. When it was applied for 3,5 minutes, temperature change was 20°C. Even the application duration was too short; temperature change was high with this output power. Therefore, temperature measurement in the presence of ICG was found unnecessary.

Table 6.4
Temperature change after the laser application in the presence of ICG or without ICG.

Output Powers (mWatt)	Exposure Duration (Seconds)	ICG (+/-)	Temperature Change
300	30 minutes	+	6,41°C
300	30 minutes	-	1,08°C
500	15 minutes	+	7,68°C
500	15 minutes	-	1,32°C
900	3,5 minutes	-	20,2°C
900	75 seconds	-	15,35°C

6.3.3 Antibacterial effect of PDT on excisional wounds infected with *S. aureus* 1755

Pre-dosimetry studies with *S. aureus* 1755 on excisional wounds were started with 200 mW of output power applying for 15 minutes. Administered ICG concentrations with this light dose were 4, 10, 25, 50, and 75 μg/ml. These combinations were not succeeded to decrease bacterial cell viability in the wounds and mostly gave results same as in control group. In some groups, amount of bacterial colonies was twice as in control groups, as shown in table 6.5.

Table 6.5
Bacterial cell count after PDT applications on excisional wounds infected with *S. aureus* 1755.

Output powers (Watt)	Exposure duration (seconds)	Energy dose (J/cm^2)	ICG concentration (µg/ml)	Cell count (CFU/gr)
Control group			-	$1,9x10^6$
0,2	900	231	10	$4x10^6$
			25	$1,85x10^6$
			50	$1,44x10^6$
			75	$2x10^6$

After these disappointing results, output power, exposure duration and ICG concentrations were changed to obtain bactericidal effect. All of these combinations were not sufficient to diminish bacterial burden in the excisional wounds. Around 90% decrease was observed only in some groups with higher output powers, but these results were not consistent. They were obtained in groups with higher output powers, which may result in thermal damage of the target tissue. Applied energy dose was quite high, but it was not sufficient to kill the bacteria inside the wound. Applied parameters and viable cell count after applications were summarized in table 6.6.

Table 6.6
Viable cell count in control group (on average) was $1{,}5x10^5$ CFU/gr. Antibacterial effect of PDT application was shown in percentage.

Output Powers (Watt)	Exposure duration (seconds)	Energy Dose (J/cm^2)	ICG concentration (µg/ml)	Cell viability (CFU/gr)	Antibacterial effect
0,9	75	84	4	$5{,}4x10^4$	64% decrease
			6	$1{,}2x10^5$	20% decrease
			10	$8{,}8x10^4$	41% decrease
			40	$2x10^4$	86% decrease
			50	$1{,}2x10^4$	92% decrease
			100	$2x10^4$	86% decrease
			250	$3{,}6x10^4$	76% decrease
			400	$4x10^4$	73% decrease
			500	$5{,}9x10^4$	60% decrease
0,5	300	192	500	$7{,}9x10^4$	47% decrease
0,5	600	384	1000	$2{,}4x10^5$	60% increase
0,3	1800	692	1000	$2{,}3x10^5$	53% increase
			1500	$2{,}8x10^5$	115% increase
0,2	1800	462	1000	$1{,}5x10^5$	No effect

6.3.4 Antibacterial effect of PDT on infected abrasion wounds infected with *S. aureus* 1755

Disappointing results of PDT applications on excisional wounds lead us to investigate different wound models, which have less bleeding. Abrasion wounds were established for this reason and different PDT parameters were assessed on them.

450 J/cm^2 of laser energy dose were applied together with 500, 1000 and 2000

μg/ml of ICG concentrations on infected abrasion wounds. As shown in Figure 6.8, a significant reduction in cell viability was observed in PDT groups. It was between 85-90% and corresponds to 1-2 logarithmic decrease in CFU/gr. In laser group, which only received laser irradiation, viable cell count after irradiation was nearly same as in control group. In ICG groups, which only received ICG without illumination, bacterial cell count has decreased when compared with control group. This reduction was not more than 40%. The data in this group was not significantly different from the data in control and laser groups. As expected, PDT groups were significantly different from all the other groups. But PDT groups were not significantly different from each other, showing that decrease in cell viability did not depend on ICG concentration.

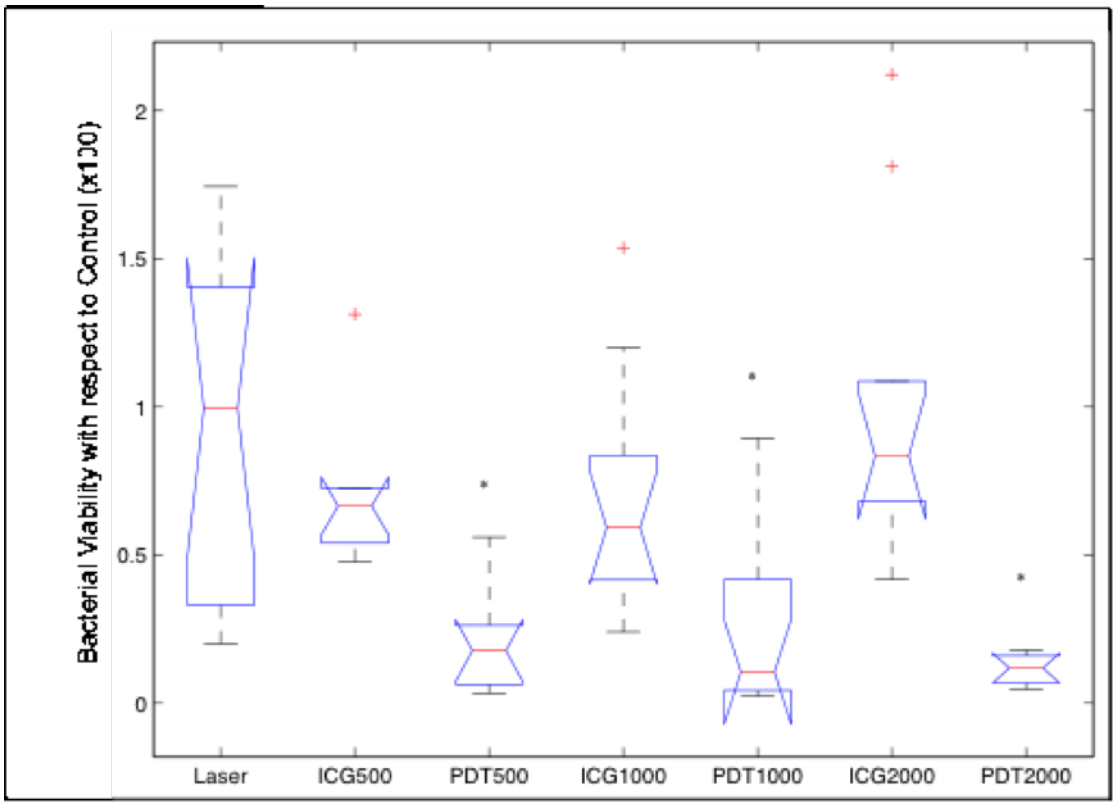

Figure 6.8 Bacterial cell viability on abrasion wounds infected with *S. aureus* 1755 after Laser, ICG and PDT applications. Laser output power was 500 mWatt, irradiation time was 15 minutes, ICG concentrations used were 500, 1000 and 2000 μg/ml. $*$ represents the statistical difference with respect to control ($p<0,05$). $n\geq 8$ number of wounds in each group.

6.3.5 Effect of different ICG concentrations on abrasion wounds infected with *S. aureus* 1755

Cytotoxicity of ICG was assessed on abrasion wounds infected with *S. aureus* 1755 without any laser application. 500, 1000, and 2000 μg/ml of ICG concentrations were used for this purpose. As seen in figure 6.8, maximally around 20-30% decrease in cell viability was observed in these ICG-only groups. These were not significantly different from Laser-only and control groups. ICG has no important cytotoxicity on bacterial cells because of topical applications on wounds.

6.3.6 Antibacterial effect of PDT on infected excisional wounds infected with *P. aeruginosa* ATCC 19660

Pre-dosimetry studies with *P. aeruginosa* ATCC 19660 on excisional wounds were started with 200 mW of output power applying for 25 minutes. Administered ICG concentrations with this light dose were 100, 150, and 250 μg/ml (Table 6.7).

Table 6.7
Bacterial cell count after PDT applications on excisional wounds infected with *P. aeruginosa* ATCC 19660.

Output Powers (Watt)	Exposure duration (seconds)	Energy dose (J/cm^2)	ICG concentration (µg/ml)	Cell count (CFU/gr)
Control group			-	$3{,}6\text{x}10^6$
0,2	1500	385	150	$4{,}8\text{x}10^6$
			200	$4{,}25\text{x}10^6$

These combinations were not sufficient to decrease bacterial cell viability in the wounds and mostly gave results same as in control group.

Later, parameters shown in table 6.8 were investigated on excisional wounds. All

of these combinations were not sufficient to diminish bacterial burden in the excisional wounds.

Table 6.8
Viable cell count in control group (on average) was 4,9x10^5 CFU/gr. Antibacterial effect of PDT application was shown in percentage.

Output powers (Watt)	Exposure duration (seconds)	Energy dose (J/cm^2)	ICG concentration (μg/ml)	Cell count (CFU/gr)	Antibacterial effect
0,9	210	252	150	1,2x10^5	75% decrease
			500	1x10^5	79% decrease
			750	1,9x10^5	61% decrease
			1000	7x10^5	42% increase
			1500	2x10^5	59% decrease
0,5	900	577	1000	7,4x10^4	84% decrease
0,3	1800	692	1000	6,2x10^5	26% increase

6.3.7 Antibacterial effect of PDT on infected abrasion wounds infected with *P. aeruginosa* ATCC 19660

Later, abrasion wounds infected with *P. aeruginosa* ATCC 19660 were established and 450 J/cm^2 of laser energy together with 1000 and 1500 μg/ml of ICG concentrations were assessed to kill *P. aeruginosa* strain. There was a slight decrease around 20% in PDT groups, unfortunately these combinations were not sufficient to consider them antibacterial. Even so, they were statistical different from other groups. In laser-only group, there was an increase around 20% in cell viability. But it was not a statistical significant result (Figure 6.9).

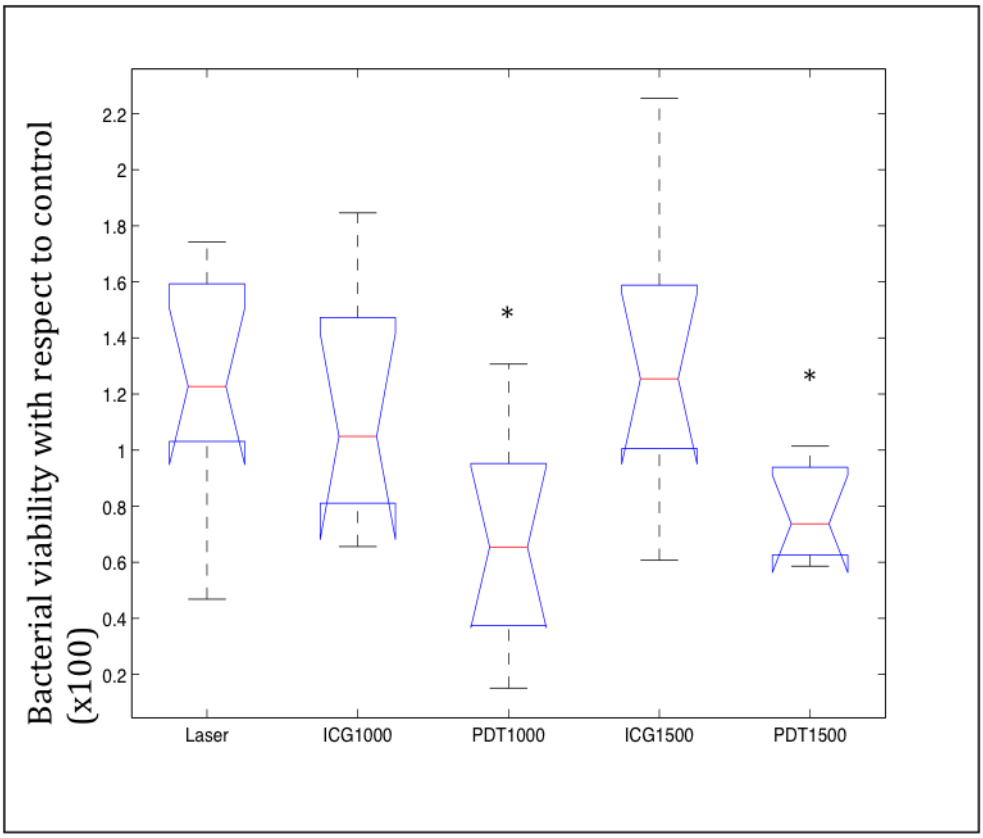

Figure 6.9 Bacterial cell viability on abrasion wounds infected with *P. aeruginosa* ATCC 19660 after Laser, ICG and PDT applications. Laser output power was 500 mWatt, irradiation time was 15 minutes, ICG concentrations used were 1000 and 1500 μg/ml. $*$ represents the statistical difference with respect to control ($p<0,05$). $n\geq 8$ number of wounds in each group.

6.3.8 Effect of different ICG concentrations on abrasion wounds infected with with *P. aeruginosa* ATCC 19660

Cytotoxicity of ICG was assessed on abrasion wounds infected with *P. aeruginosa* ATCC 19660 without any laser application, too. 1000, and 1500 μg/ml of ICG concentrations were used for this purpose. As seen in figure 6.9, application with 1000 μg/ml of ICG resulted in same bacterial viability as in control group. An increase around 20% was observed after treatment with 1500 μg/ml of ICG.

6.3.9 Wound healing process

Figure 6.10 shows the percentage reduction in size of the wounds during 11-day healing process. PDT parameters used in this wound healing process were 450 J/cm^2 of energy dose and 500 μg/ml of ICG. In first 2 days, wound area decreased nearly 40%. Then the healing process slowed down for 1-2 days, then it accelerated again. After 5th day, the size of the wounds decreased more than 50%. At 11th day, wounds were barely visible and their sizes approached nearly to zero.

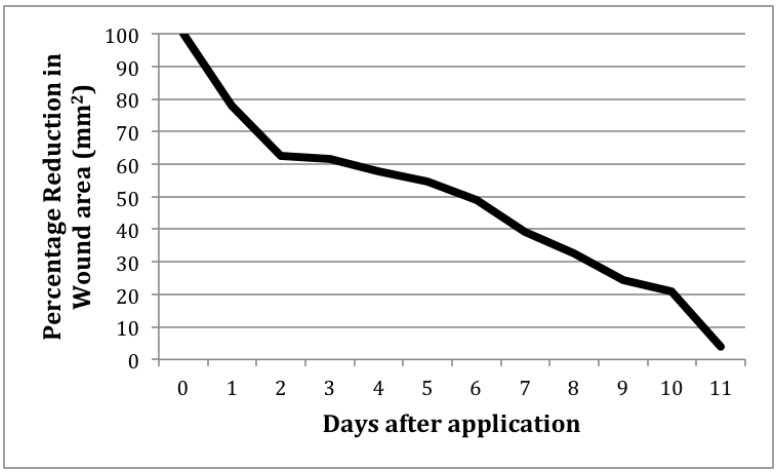

Figure 6.10 The percentage reduction in wound size after PDT application.

6.3.10 Histological analysis

As shown in figure 6.11.a, disrupted epithelial lining can be clearly observed on newly opened wound. Integrity of the epidermis was destroyed because of the scratches of the needles. To assess any thermal damage due to laser irradiation, tissue samples was removed after PDT application. Figure 6.11.b shows this tissue sample in which epithelial lining was disrupted as in tissue sample shown in figure 6.11.a. Even though high concentration of ICG was used and it was increasing the absorbance of the laser energy by tissue, there was not any observed thermal destruction. In figure 6.11.c, wound sample removed at day 2 was shown. Tissue was covered with a thick eschar. Beneath the eschar, epithelial lining which became thicker was observed. The integrity of the epidermis was recovered, but not uniform. The number of the fibroblast

increased and they concentrated at the edge of the wound. At day 4, it was observed that the eschar of the wound almost completely pulled away. But some remnants were still present. The epithelial lining became thicker, hereby integrity of epidermis was provided. Fibroblast cells at the edge of the wound were still high in number (Figure 6.11.d). At day 7, eschar on the wound completely pulled away. There was not any remnant of it. Epithelial lining started to become thinner than it was at day 4. Integrity of the epidermis was still preserved and the number of fibroblast cells decreased. Wound healing process was almost completed (Figure 6.11.e). At day 11, the scar of the wound was nearly invisible. Wound healing process was completed in 11 days as shown in figure 6.11.f. Epithelial lining reached to its normal thickness. Integrity of the epidermis was uniform. Fibroblast cells were still there, but less.

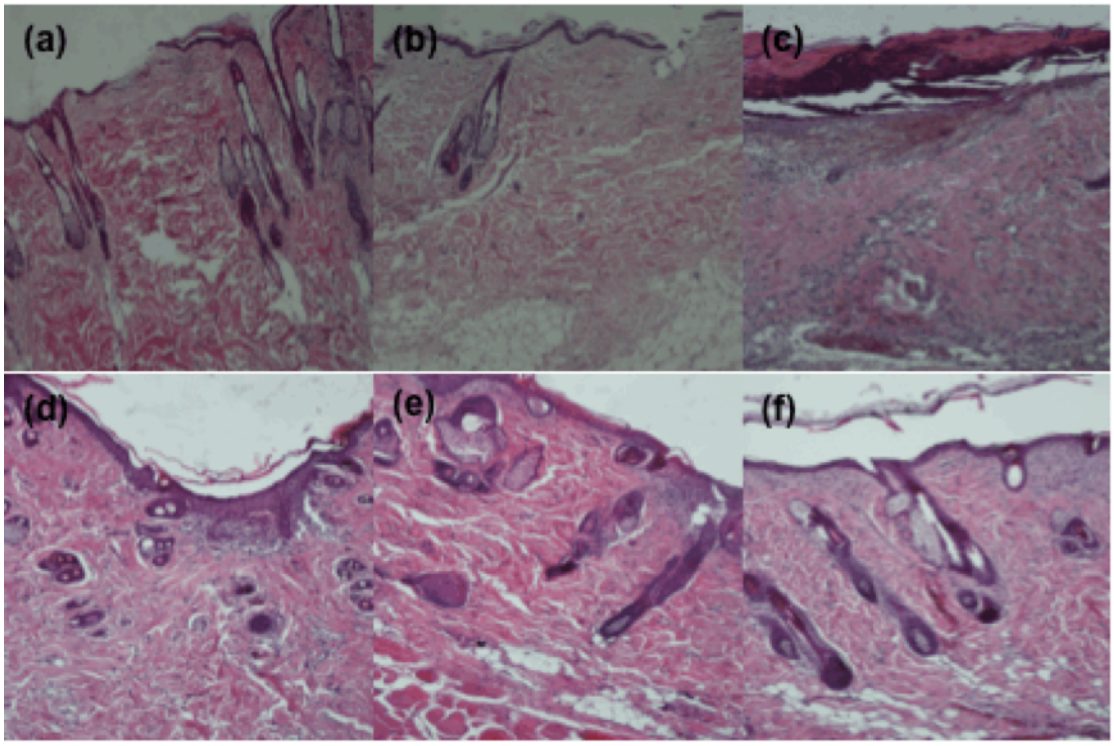

Figure 6.11 Histological image of a) wound which was newly opened and yet not received ICG or Laser (magnification (mag): x100), b) wound which was immediately removed after PDT application (mag: x100), c) PDT treated wound which was removed at 2nd day after application (mag: x100), d) PDT treated wound which was removed at 4th day after application (mag: x100), e) PDT treated wound which was removed at 7th day after application (mag: x100), f) PDT treated wound which was removed at 11th day after application (mag: x100).

Figure 6.12 shows the wound morphologies during healing process. The sample in figure 6.12.a is a newly opened wound and the sample in figure 6.12.b is a PDT-

treated sample. PDT-treated sample had eschar on it at 2nd day (Figure 6.12.c). The eschar on the wound diminished at 4th day (Figure 6.12.d). The eschar totally disappeared, there was only small redness, and the wound size remarkably decreased at 7th day (Figure 6.12.e). There was any eschar or redness, only small scars in the place of the wound at 11th day (Figure 6.12.f).

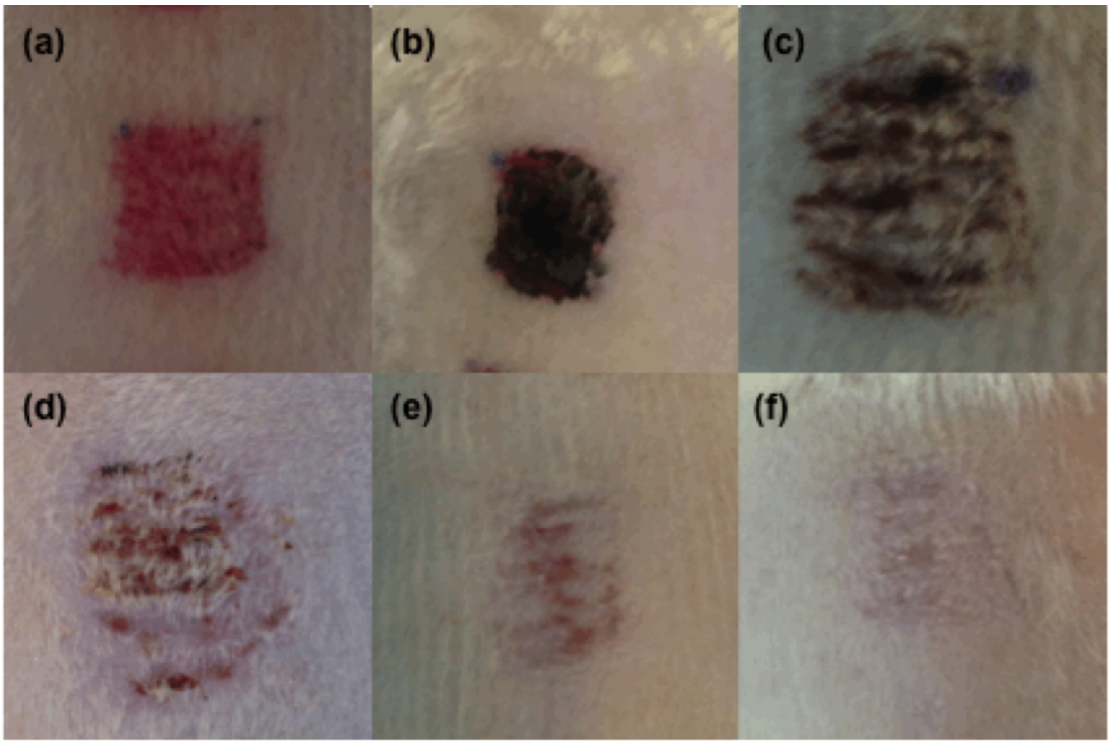

Figure 6.12 Wound appearance of a) a sample which was newly opened and yet not received ICG or Laser, b) sample which was immediately removed after PDT application, c) PDT-treated sample which was removed at 2nd day after application, d) PDT-treated sample which was removed at 4th day after application, e) PDT-treated sample which was removed at 7th day after application, f) PDT-treated sample which was removed at 11th day after application.

6.4 Discussion

Photodynamic therapy is regarded as a promising new antibacterial method and there are several studies about photodynamic therapy using visible light and different photosensitizers. Researchers have generally confused on investigating more successful photosensitizers, which have higher affinity to bacterial cells to obtain better bactericidal effect [6, 74, 75, 76, 77, 78, 79]. In this study, near-infrared spectrum was chosen to take advantage of deeper penetration capability through biological tissue [25]. The

suitable photosensitizer for 808-nm is indocyanine green and it has some disadvantages when applied on bacterial cells. It has anionic chemical structure and relatively big size, which affects the interaction possibility of this molecule with bacteria and diffusing through the cell wall [34, 35, 36, 37, 38, 58, 80]. In addition, ICG molecules have the capability to bind plasma proteins in 3-4 minutes [81]. This situation makes ICG molecules to lose the activity of absorbing enough light and subsequently to produce efficient reactive oxygen species for eliminating the bacteria. For this reason, abrasion wound model, which has pretty low bleeding, was used to eliminate the possibility of binding plasma proteins to ICG molecules in this study. In addition, ICG solution was added to the wound with intervals of 3 minutes until the total volume of 50 μl (5x10 μl) was completed during laser application. Refreshing the photosensitizer on the wound in every 3-minute was thought to decrease the possibility of binding plasma protein and increase the possibility of absorbing light and subsequent reactive oxygen production.

PDT application with laser energy dose of 450 J/cm^2 and ICG concentrations of 500, 1000, and 2000 μg/ml by applying ICG as described above resulted in significant reduction of bacterial cell count. Actually effects of these concentrations were not significantly different to each other. In order to diminish the effect of higher concentration of ICG, 500 μg/ml was chosen to observe the effect of PDT during healing process.

Success of the treatment was clearly observed on the images of histological specimens. There was not any observed thermal damage immediately after laser irradiation or inflammatory reaction during healing process. Organism has recovered from infection after treatment in a very short span of time. Healing period of a superficial wound is known to be between 15-21 days. Dai et al also studied abrasion wound model infected with *S. aureus* on mice. They showed that 90% of 1 cm2 of wound area was healed in 11 days [14]. In our study, nearly 100% of 1 cm^2 of wound area was healed in 11 days. 808-nm of light and ICG achieved faster healing process on the same infected wound model.

A possible important effect of irradiation with 808-nm diode laser was heating

effect and consequently higher temperature increase that may result in thermal damage. Passing the tissue temperature beyond 45°C causes irreversible tissue damage. This is an unwanted result during healing period of the biological tissue. Illumination with output power of 500 mW for 15 minutes in the presence of ICG caused approximately an increase of 8°C on average and the total tissue temperature was still below critical point for irreversible tissue damage. Histological analyses also confirmed these results showing that there was not any thermal damage in the target or neighboring tissue.

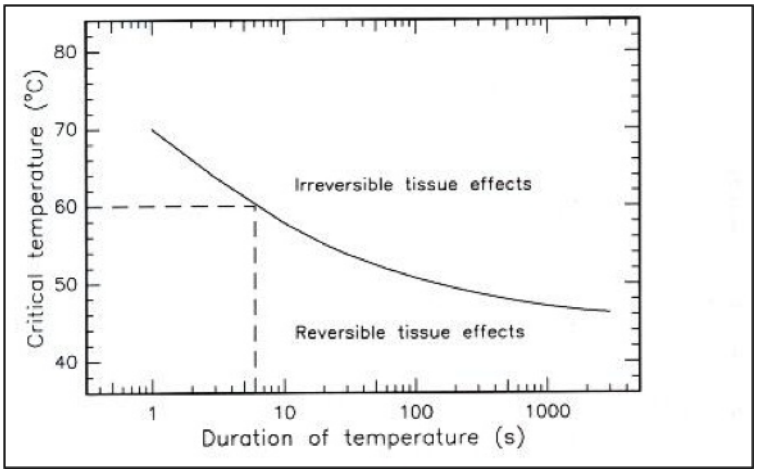

Figure 6.13 Graph of critical temperatures that cause cellular necrosis [82]

Wounds infected with *P. aeruginosa* was examined same as the wounds infected with *S. aureus*. First, excisional wounds were created and infected with *P. aeruginosa* strain. After unsuccessful applications with lower output powers, exposure durations, energy dose, and ICG concentrations were changed to succeed eradication of this strain. These modifications generally caused proliferation of *P. aeruginosa* instead of eradication. These results brought to mind the risk of biostimulation as we found it in *in vitro* studies with this bacterium. Lower laser energy doses may stimulate some cellular mechanisms that are responsible for cell survival. This may be the reason for proliferation result of excisional wounds. But this situation has not been examined afterwards. The aim was to find antibacterial doses of PDT. Wound model was changed to abrasion wound and ICG application to the wounds has been changed, too. ICG solution was added to the wound with intervals of 3 minutes until the total volume of 50 μl (5x10 μl) was completed during laser application. Unfortunately, these interventions were

not sufficient to destroy *P. aeruginosa* efficiently. In *in vitro* studies, we had to apply three times higher energy dose and ICG concentrations to kill *P. aeruginosa* when compared to energy dose and ICG concentrations used for *S. aureus*. Cell wall structure of gram-negative bacteria makes eradication by this method more difficult. Applied doses in *in vivo* studies were quite high. Therefore we do not want to increase them more, which may result in damage of neighboring tissue. To eliminate this problem, ICG application to the wounds may be changed to improve the interaction between ICG molecules and bacterial cells within the tissue.

6.5 Conclusion

In this study it was shown that proposed modalities of PDT were successful in rapid eradication of viable bacterial cells, and accelerated healing process. This method was also known to be advantageous not to cause any bacterial resistivity like antibiotics. However, these results may not be valid for gram-negative bacteria. Cell wall structure of gram-negative bacteria may obstruct the efficiency of 808-nm diode laser and indocyanine green. Therefore this application has to be investigated for different kinds of bacteria and has to be improved and modified to eradicate gram-negative strains.

7. OVERALL DISCUSSIONS AND CONCLUSIONS

Photodynamic therapy has been accepted as a promising treatment tool for many oncological and non-oncological diseases [1, 24]. It has been widely investigated to treat different kinds of cancerous tissues, and it has been started to use in treatment of many malignancies in clinics [7, 35, 36, 37, 39, 40, 41]. Despite, it has become a standard protocol to treat some ophthalmic and dermatological diseases, such as macular degeneration and acne vulgaris [7, 8]. There are many considerations to improve this method and researches still continue as a hot topic.

By now, mostly visible spectrum has been preferred and investigated for antibacterial PDT applications. Cationic photosensitizers, which are suitable for visible spectrum, have many advantages over anionic and neutral ones, depending on the chemical structure of them and bacterial cell wall [18, 22, 26, 27, 28, 29, 79]. Therefore near-infrared light has not been preferred, because of some disadvantages of the photosensitizer (ICG) that is suitable for this spectrum. Anionic structure of ICG and its relatively big size prevent it to interact with bacterial cells [58, 80]. However the pattern of near-infrared light in biological tissue may make it more advantageous for PDT applications. Its deeper penetration capability in tissue gives the opportunity to treat problematic cells, which are located deep inside the tissue [25, 30, 31, 32, 33, 58].

In vitro study explained in Chapter 2 has showed high capacity of 809-nm laser light and ICG combination to treat gram-positive and even gram-negative bacteria with quite low energy dose and ICG concentrations. First, dosimetry studies were done to find optimum parameters to destroy wild type strains. Efficient bactericidal effect to eradicate 99% of bacterial burden was achieved with lower energy dose and ICG concentration compared to the study performed by Omar et al [9]. There were some basic differences between these two studies. The strains of the bacteria and the output power were different in these studies. These may be the reason to obtain same bactericidal effect with different energy doses and ICG concentrations. The other concern in this

study is the differences in reaction of bacteria to PDT depending on whether they are gram-positive (*S. aureus*) or gram-negative (*P. aeruginosa*). To destroy *P. aeruginosa* efficiently, we had to apply higher energy dose and ICG concentrations compared to *S. aureus*. This condition depends on the structural changes of bacterial cell wall that was explained in chapter 2 in detail. Cell wall orientation of gram-negative bacteria is tighter which makes the interaction of photosensitizer and bacteria more difficult. The final concern in this study was to show strain-dependent PDT effect. Optimum PDT parameters that destroyed wild-type strains of *S. aureus* and *P. aeruginosa* were investigated to find out their effects on resistant strains of the same bacteria. Actually same energy dose with lower ICG concentration has worked on different strains. It was thought that this must be related to the structural differences of cell wall in different strains of the same bacteria. There are some studies, which show the difference of optimum parameters to kill different strains, but not affected by the resistivity mechanism of these strains [27, 29, 53].

In Chapter 3, the effect of output power of 809-nm diode laser was analyzed. In the first part of this study, the output power used was 1 Watt. Generally, lower output powers were used in antibacterial PDT studies, such as the study in that near-infrared light source was used Omar et al did [9]. Main behavior of this spectrum in biological tissue is photothermal. But we did not want any photothermal effect in this study; only want the photochemical mechanism of photodynamic therapy, which produces reactive oxygen species. Thus, effect of output power in this *in vitro* study was investigated by changing power, but without changing energy dose. Exposure duration of the cells to the light was changed to obtain same energy dose. Increasing or decreasing output power made no significant difference in the result of Laser-only or PDT application. We understood that powers around 1 Watt did not cause any bacterial cell death depending on thermal effect during PDT applications *in vitro*.

During dosimetry study of PDT on *P. aeruginosa* strain, it was started to apply lower energy dose and ICG concentrations that were efficient to kill *S. aureus* strain. These parameters could not destroy *P. aeruginosa* strain, rather an increase in viable cell count after these applications was observed in the first experiments. These results

lead us to investigate risk of proliferation when parameters are not well optimized during PDT applications. After increasing the number of data, it was clearly seen that first proliferation data was consistent with the data of whole experiments. Nearly 60% proliferation was observed at low dose and ICG concentration. While increasing the dose and concentration, proliferation percentage decreases. At some point, application became neutral without any proliferative or destroying effect. Later, antibacterial effect could be seen after increasing dose and concentration high enough. Actually this condition was explained with photooxidative mechanism. Primary products of mechanism of photodynamic therapy that kill target cells are reactive oxygen species and The amount of oxygen radicals generated depends on the concentration of drug, light dose and the amount of molecular oxygen that are available in the environment [7, 18, 21, 57]. As explained in chapter 4, lower concentration of reactive oxygen species can stimulate different biochemical pathways in the cells causing cell survival and proliferation [63, 64, 65, 66, 67, 68, 69, 70]. In this study, it was understood that lower concentration of ICG with lower light dose caused bacterial proliferation instead of cell death. Increasing dose and ICG concentration increased the amount reactive oxygen species produced, and hence antibacterial effect could be observed. This study strongly emphasized the importance of dosimetry in PDT applications. Proliferation of unwanted cells may happen instead of destroying them if laser dose, application duration or photosensitizer concentration are not well optimized.

In Chapter 5, this method was examined on rat wound model. First, excisional wound model was created and infected with resistant strains of bacteria. Antibacterial effect of this modality was investigated *in vivo* starting with optimum parameters found in *in vitro* experiments. Laser dose and ICG concentration that were efficient in *in vitro* study were not efficient to destroy bacteria on animal wound model. It was an expected situation because of the complexity of an organism. Later, parameters were gradually increased to find out sufficient doses and concentrations. Unfortunately, applied maximum light dose and ICG concentration that were not toxic or hazardous for healthy tissue could not treat infected wound. Meanwhile the study of Kirchherr et al was found during literature survey. They said in their study that ICG molecules have the capability to bind plasma proteins in 3-4 minutes [81]. Actually, excisional

wounds have quite high bleeding. It was thought that high concentration of plasma proteins in excisional wounds might be the reason of these unsuccessful results. So it was decided to change the wound model. Later, abrasion wounds, which have very low bleeding, was created and infected with resistant *S. aureus* and *P. aeruginosa*. Mode of ICG application was changed, too. ICG was applied as 5 parts with 3-minutes interval to prevent its ability to bind plasma proteins and refresh the photosensitizer solution on the wounds. Optimum parameters to treat abrasion wounds infected with *S. aureus* strain could be found out. More than 90% bacterial burden was destroyed. Healing process of these wound were examined and observed that these wounds healed in shorter time period than expected. When these wounds were investigated histologically, recovery could be examined in detail. It was clearly seen that there was not any thermal damage depending on the laser application. We concluded that abrasion wounds infected with *S. aureus* could be successfully treated with this modality.

Same parameters could not be sufficient to treat abrasion wounds infected with resistant *P. aeruginosa*. Quite high laser dose and ICG concentrations were applied on these wounds, but bacterial burden could not be diminished. Laser dose could not be increased further not to cause any thermal damage, and ICG concentration could not be increased further not to cause any cytotoxicity. This situation might arise from the cell wall orientation of gram-negative bacteria and anionic nature of ICG molecule. Thus, some other ways to administer ICG solution to abrasion wounds infected with *P. aeruginosa* have to be investigated to eliminate its disadvantage of being anionic as a future work, such as Kirchherr and his colleagues described in their study [81]. Formation of a micellar system for ICG molecule may increase the possibility of this molecule to interact with gram-negative bacteria, which in turn may result in better bactericidal effect.

As a conclusion; it was seen that this treatment protocol has many advantages to eradicate infection rapidly, to decrease time period for wound healing process, to prevent the development of antibiotic-resistivity of bacteria and to destroy antibiotic-resistant strains easily. Still further studies are needed to improve application modalities to be successful in destroying gram-negative bacteria efficiently. After eliminating

the disadvantages depending on anionic nature of photosensitizer and cell wall orientation of gram-negative bacteria, PDT application with near-infrared light and ICG will be a promising and powerful tool to treat chronic wound infections with shorter healing time period and minimum side-effects, such as thermal damage.

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