EVALUATION OF LIGHT AND CONCENTRATION DOSE ON CELL VIABILITY AT PHOTODYNAMIC THERAPY *in vitro*

by

NURAY AYSAN

B.Sc. in Chemical Engineering, Yildiz Technical University, 2009

Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Engineering

Boğaziçi University
2014

ACKNOWLEDGMENTS

I would like to thank my supervisor Prof. Murat Gülsoy, for his kind academic support and his encouraging guidance to me. In addition to this, I would like to thank him for his ability about looking events from different points to search new related sides of this study which me makes better researcher and for his confidence on me which encourages me against difficulties during the this study.

I would like to thank Dr. Şahru Yüksel for her contributions to make this study possible in Molecular Biology and Genetics Department and members of AKIL Laboratory for their supports.

I would like to thank Assist. Prof. Dr. Bora Garipcan for being member of my thesis committee.

I would like to thank Assist. Prof. Dr. H. Özgür Tabakoğlu for being member of my thesis committee.

I would like to give my special thanks to research assistant Nermin Topaloğlu from Biophotonics Laboratory. I appreciate her because of her academic and kind motivation support and friendship during this study.

This study was supported by Bogazici University Research Projects (BAP Project No: 5892). So I am grateful to Bogazici University for their Support.

And thanks to my best friends Merve Kaplan, Gizem Sınmaz and Pınar Öz. I made through the difficulties and worked effectively during this study. I would like to thank them for motivation support and kind helps and encouragement.

And my friends, I would like to thank Ayşegul Şen, Ebruhan Akgüller, Tuğba Köker and Ayşe Sena Kabaş Sarp for their nice motivation support, kind friendship and nice patience. Especially I would like to thank all of them for encouragements. Because when I was so tired, they could work more efficacy with their nice confidence on me. Thank you my friends.

ABSTRACT

EVALUATION OF LIGHT AND CONCENTRATION DOSE ON CELL VIABILITY AT PHOTODYNAMIC THERAPY *in vitro*

Photodynamic Therapy is a promising and safe antimicrobial treatment that includes in a chemical agent, called a photosensitizer, which is activated by appropriate light energy and it results in production of reactive oxygen species (ROS) which have an important role in destroying the target cells. PDT dosimetry (light dose, photosensitizer dose and concentration of produced ROS) is very critical in the photoactivation process. Low concentration of oxygen radicals or low level light may cause cell proliferation with some biochemical pathways instead of the killing effect of antibacterial PDT. For this reason, there is a biostimulation risk during antibacterial PDT and optimization of PDT dose properly is very important to overcome the multidrug resistant bacteria problem on wounds. The main purpose of this study was to investigate the PDT safe region for bactericidal application and to demonstrate the importance of PDT dosimetry. In this study, PDT with different concentrations of indocyanine green (ICG) (20, 50, 100, 125, 150, 200 and 250 μg/ml) and different doses of 809-nm diode laser (84, 168 and 252J/cm^2) was investigated on Pseudomonas aeruginosa ATCC 27853 in vitro for PDT safe region. In this study, the cell proliferation of P. aeruginosa strain was observed instead of the PDT killing effect, when 84 J/cm^2 of energy dose (809-nm diode laser) was applied with 20, 50, 100, 125 and 150 μg/ml of ICG concentrations. When we increase the energy doses with the same concentrations, at optimum higher concentrations, the PDT killing effect was significantly observed (150 μg/ml ICG with 168J/cm^2 and 125 μg/ml ICG with 252 J/cm^2). The results of experiments show that there could be biostimulation on pathogens if PDT dosimetry is not optimized properly.

Keywords: Photodynamic therapy, 809-nm diode laser, indocyanine green, P. aeruginosa, cell proliferation, biostimulation.

ÖZET

HÜCRE CANLILIĞI ÜZERİNDE IŞIK VE KONSANTRASYON DOZUNUN FOTODİNAMİK TERAPİDE *in vitro* OLARAK DEĞERLENDİRİLMESİ

Fotodinamik Terapi ümit verici ve güvenilir bir antimikrobiyel tedavi yöntemidir. Bu yöntem,uygun dalga boyunda ışıkla aktif hale gelen ışığa duyarlı kimyasal kullanımını içerir. İlaç ile ışığın etkileşimi hedef hücrelerin ölmesinde önemli bir role sahip olan reaktif oksijen türlerinin oluşmasına neden olmaktadır. PDT dozu (ışık dozu, ilacın dozu ve üretilen ROS konsantrasyonu) fotoaktivasyon prosesinde çok kritiktir. Oksijen radikallerinin düşük konsantrasyonları ya da düşük miktarda ışık,antibakteriyel PDT'nin öldürücü etkisi yerine bazı biyokimyasal yollarla hücre çoğalmasına neden olabilmektedir.Bu nedenle,antibakteriyel PDT süresince biyostimülatif etki vardır. PDT dozunun uygun düzeyde optimizasyonu, yaralardaki enfeksiyonlara neden olan antibiyotik direnci olan bakterilerin tedavi etmekte çok önemlidir. Bu çalışmanın temel amacı, bakteri öldürücü uygulamalar için PDT güvenli alanı incelemek ve PDT dozunun önemini göstermektir. Bu çalışmada, PDT'nin öldürücü etkisinin olduğu alanı tespit etmek için değişik konsantrasyonlarda (20, 50, 100, 125, 150, 200 and 250 μg/ml) indosiyanin yeşili (ICG) ile 809-nm diyot laserin çeşitli enerji dozlarında (84, 168 and 252J/cm^2) *Pseudomonas aeruginosa* ATCC 27853 bakterisi üzerinde in vitro olarak uygulanmıştır. Bu çalışmada, 809-nm diyot laserin 84 J/cm^2'lik enerji dozu ile ICG'nin 20, 50, 100, 125 and 150 μg/ml dozları uygulandığında, *P. aeruginosa* bakterisinde PDT etkisi yerine bakteri sayısının çoğaldığı gözlemlenmiştir. Aynı konsantrasyon oranlarında laser enerji dozu arttırıldığında, daha yüksek optimum konsantrasyonlarda PDT'nin öldürücü etkisi anlamlı şekilde elde edilmiştir (150 μg/ml ICG ile 168J/cm^2 enerji dozu, 125 μg/ml ICG ile 252 J/cm^2 enerji).Deney sonuçları göstermektedir ki PDT dozu iyi ayarlanmazsa,patojenlerde biyostimülatif etki meydana gelebilir.

Anahtar Sözcükler: Fotodinamik terapi, 809-nm diyot laser, indosiyanin yeşili, Pseudomonas aeruginosa, hücre çoğalması, biyostimülatif etki

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LIST OF ABBREVIATIONS

ALA	δ-aminolevulenic
ATP	Adenosine triphosphate
C	control group
CFU	colony-forming units
cm^2	centimeter square
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
Hp	Hematoporphyrin
HPD	hematoporphrin derivative
ICG	Indocyanine Green
J	joule
LLLT	low level light therapy
mL	mililiter
Near-IR/NIR	Near Infrared
nm	nanometer
PBS	phosphate buffer saline
PDT	Photodynamic Therapy
PpIX	Protoporphyrin IX
PS	Photosensitizer
PS*	triplet-excited state photosensitizer
ROS	Reactive Oxygen Species
S	organic substrate
UV	ultraviolet
W	watt
μg	microgram

1. INTRODUCTION

In recent years, wound infections are a continuing major problem all around the world. Apart from the fact that infected wounds cause morbidity and mortality, the rapid increase of multi-drug resistant bacteria requires urgently a new treatment for infectious diseases such as those resulting from burn wounds. Many studies in vitro and in vivo show that Photodynamic Therapy (PDT) is an effective method to destroy microorganisms by combining a chemical dye and a light source application on bacteria. For this reason, scientists reconsider antibacterial PDT as an alternative antimicrobial method against life threatening bacteria that have antibiotic resistance for example gram-negative bacterium Pseudomonas aeuruginosa [[1], [2], [3], [4], [5]].

Antibacterial photodynamic therapy can be used as an efficient treatment method to kill multi-drug resistant bacteria causing infections since PDT is more advantageous than antibiotics [6]. It is a safe treatment because the harmless suitable wavelength of light applied on bacteria is harmless and the chemical dye, called photosensitizer, is non-toxic. Light activates the photosensitizer and it leads to produce reactive oxygen species (ROS) that are responsible for killing microorganisms. In addition to this, bacteria cannot develop resistance like bacteria develop resistance against antibiotics with repeated use [3], [7], [8], [9], [10].

The PDT mechanism is obtained by Type I and Type II reactions which damage biomolecules in cell membranes of bacteria thanks to oxygen molecules in the PDT process. The cell membranes of bacteria are destroyed by free oxygen radicals which are formed when light activates the phototsensitizer. In Both Type I and Type II, energy transfers from ground state to singlet-excited state and then to the triplet state. In Type II, obtained singlet oxygen molecules react with various biological molecules because of transforming energy transfer from triplet state photosensitizer to ground state molecular oxygen. In Type I, triplet excited state leads an electron interaction with neighboring molecules, which cause the formation of free oxygen radicals that

have reactions with oxygen like hydrogen peroxide and hypochlorite radicals. These oxidants have the impact of killing the bacteria in infected wounds [11],[12], [13], [14], [15], [16], [17].

As a photosensitizer, ICG has been used in PDT for cancer treatment since methylene blue and porphrin are used against bacteria in PDT applications in general. But ICG has an absorption peak around 800-nm. For this reason, the use of near-infrared wavelentghs with ICG in PDT applictions is the most proper to destroy bacteria in infected wounds. It leaves the body very quickly and it has no adverse effect. Near-IR wavelengths penetrate deep into tissues safely. Its possible damage to the surrounding tissues is limited. Hence, ICG and 809-nm diode laser combination in PDT is the most suitable and effective way to kill bacteria in infected wounds such as burns and deeper wounds [3], [6], [18], [19], [20].

The killing mechanism of the photosensitizer depends on the existence of oxygen molecules and the light. Besides, the efficacy of photosensitizers is different in every photoactivation process on bacteria because of the different cellular structures of bacteria. Photosensitizer absorbs the appropriate wavelength of light and it causes the energy transfer to molecular oxygen which produces highly reactive oxygen radicals [4],[21]. The susceptibility of the photoinactivation of bacteria can be different for different bacteria types due to their cell structure differences. For instance, gram-positive bacteria are surrounded by a 20-80nm thick outer wall which is separated from the cell membrane. On the contrary, gram-negative bacteria are surrounded by the presence of an additional 10-15nm thickness. At this point, the most important thing is PDT dosimetry because the determination of optimum light and photosensitizer doses has an important role in killing of bacteria causing wound infections. Producing singlet oxygen molecules during the photoactivation process are responsible for bacteria eradication and bacteria proliferation. For this reason, which photosensitizer and which light wavelength in which doses are applied on bacteria is a critical point in PDT. Singlet oxygen at lower doses can cause biostimulative effect on bacteria instead of killing effect of PDT [22], [23].

Biostimulation is a kind of biological mechanism that can be obtained by lower laser applications. Biostimulation increases cellular proliferation in different ways such as by triggering calcium channels. Additionally, biostimulation activates the cell viability at cellular level in several ways such as an increase in DNA synthesis and the production of ATP. ROS have an important role in cell proliferation as well. In lower concentrations, some biochemical processes such as cell growth, the release of transcription factors and gene expression are stimulated by oxidative stress. For this reason, ROS in lower concentrations during antibacterial PDT might lead to cell proliferation. [12], [24],[25], [26].

1.1 Motivation and Objectives

Although, PDT has more advantages in applications on bacteria compared with antibiotics, in some previous studies of our Biphotonics Laboratory indicate that lower doses might affect cell viability and result in cell proliferation which is opposite of the expected PDT killing effect on Pseudomonas aeuruginosa. Motivation of this study is to highlight the importance of PDT dosimetry including photosensitizer concentration and light dose. The optimization of PDT doses has a proper role in photoinactivation of bacteria due to free oxygen radicals of PDT, if the correct doses are applied. On the other hand, there could be biostimualtion risk when lower doses are applied due to free oxygen radicals of PDT.

The aim of this study was to demonstrate the safe PDT region of ICG-PDT application on Pseudomonas aeuruginosa and to investigate the risk of biostimulative effect on cell viability of Pseudomonas aeuruginosa because of free oxygen radicals of PDT with certain low light on bacteria. In particular, the importance of PDT dosimetry in both light dose and phosensitizer concentraion are very critical on antibacterial PDT applications in order to kill the microorganisms causing infections in wounds. Our study had the purpose of investigating the combination of light and phosensitizer doses that is critical for PDT against bacterial infections and determining PDT safe region in order to prevent from biostimulation.

1.2 Thesis Overview

In Chapter 2, the literature on Photodynamic Therapy, Wound Infecting Bacteria-Wound Infections and the Biostimulative Effect is explained in detail.

2.1 part of Chapter 2 includes explanations of the history of PDT, the mechanism of PDT, clinical Uses of PDT, antibacterial PDT, PDT dosimetry, PDT with 809-nm diode laser and ICG under the photodynamic therapy title. In 2.2, wound infecting bacteria and wound infections are explained under the subtitles of Gram+ and Gram- Bacteria, Antibiotic Resistance and Wound Infections. 2.3 part of Chapter 2 explains Biostimulative Effect via description of Biostimulation, relevance of Biostimulation and Low-Light Source and Effect of O_2 radicals in Biostimulation Mechanism.

In Chapter 3, detailed information about materials and methods used in this study are mentioned.

In Chapter 4, results of experiments done on P. Aeruginosa by using 809-nm diode laser and ICG are explained and the efficacy of PDT region was shown and details of it are explained.

In Chapter 5, results of this study are discussed by comparison with other studies.

In Chapter 6, the conclusion of the study and future studies are explained.

2. THEORY

2.1 Photodynamic Therapy

Photodynamic Therapy (PDT) is a relatively alternative medical treatment and safe application for several diseases such as skin infections, cancer, and Bowen's disease etc. due to its non-toxic effects and inexpensive price. PDT is a method which combines the use of light-activated chemical agents (called photosensitizers) with light at proper wavelength which results in the production of reactive oxygen species (ROS). The generation of reactive oxygen species (ROS) in PDT destroys the target cell components such as nucleic acids, membrane lipids and proteins. Due to the reactivity of ROS toward cell components, PDT is commonly used to kill cancer cells in clinical oncology and to treat non-malignant conditions and bacterial infections. In recent years, the increased resistance of bacteria against antibiotics has made antibacterial PDT is an effective treatment for microbial skin infections all around the world [1], [8], [27], [28].

2.1.1 History (Light Source-Photosensitizers)

The use of light as therapy originated over three thousand years ago. In ancient times, the Egyptians, Indians and Chinese people used the sunlight with natural plant extracts like chlorophylls due to its activation with light energy to treat various types of disorders such as skin cancer, rickets and psychosis etc. The Greeks used sun light to make people healthy by exposing the whole body to the sun [29], [30]. They recommended that the method is useful for 'restoration of healt' and Herodotes called it 'heliotherapy'. The use of sun exposure as therapy appears again in the 18^{th} century to treat rickets due to the increased absorption of calcium and phosphorus in the body [31]. In addition to this, Sniadecki, a Polish physician, also reported in 18^{th} century that sun exposure is important to prevent the health from rickets disease. In 1893,

Niels Finsen, a Danish researcher, used red light to treat small pox with the purpose of preventing pustule suppuration [31]. He later received the Nobel Prize in 1903 due to his work with the use of light from a carbon arc to treat skin tuberculosis lupus vulgaris [31],[32].

In 1904, Professor Herman von Tappeiner, the director of the Pharmacological Institute of the Ludwig-Maximilians University in Munich and Oscar Raab, a German medical student in Professor von Tappeiner's laboratory, described the term 'photodynamic action' ('photodynamische Wirkung') based on their observations of the combination of the light at certain wavelengths and acridine asa dye for killing Paramecium caudatum [32], [33],[34].

In 1900, Oscar Raab discovered the toxic effect of acridine combined with certain wavelengths of light on paramecia. His experiments played an important role in the discovery of photodynamic action. He used the same acridine dye concentrations with various light exposures to kill paramecia. He noticed that exposure time of light is a lethal parameter factor. Moreover he reported that paramecia in acridine solutions were killed by the exposure of sunlight, whereas they were alive for longer in sunlight when there was no acridine present. His discovery indicates that combination of light and acridine has more lethal effect on the paramecia than their appliction by themselves alone. After this study, Professor Von Tappeiner continued to investigate by himself and he discovered that the presence of oxygen is a requirement for the photodynamic effect. In 1903, H. Von Tappeiner and A. Jesionek (a dermatologist) reported that they had treated skin cancer with a combination of topically applied %5 eosine and white light, effectively [32], [33], [35], [36], [16].

Meyer-Betz, a German scientist, injected 200 mg of hematoporphyrin into his own vein in 1913. According to his report, no side effect was observed until exposure to sunlight. After he exposed himself to the light, there was pain and skin swelling on his body. The phototoxic property of porphyrin was further explored by Policard [36]. In 1933, a book including several papers about UV light for treatments of some

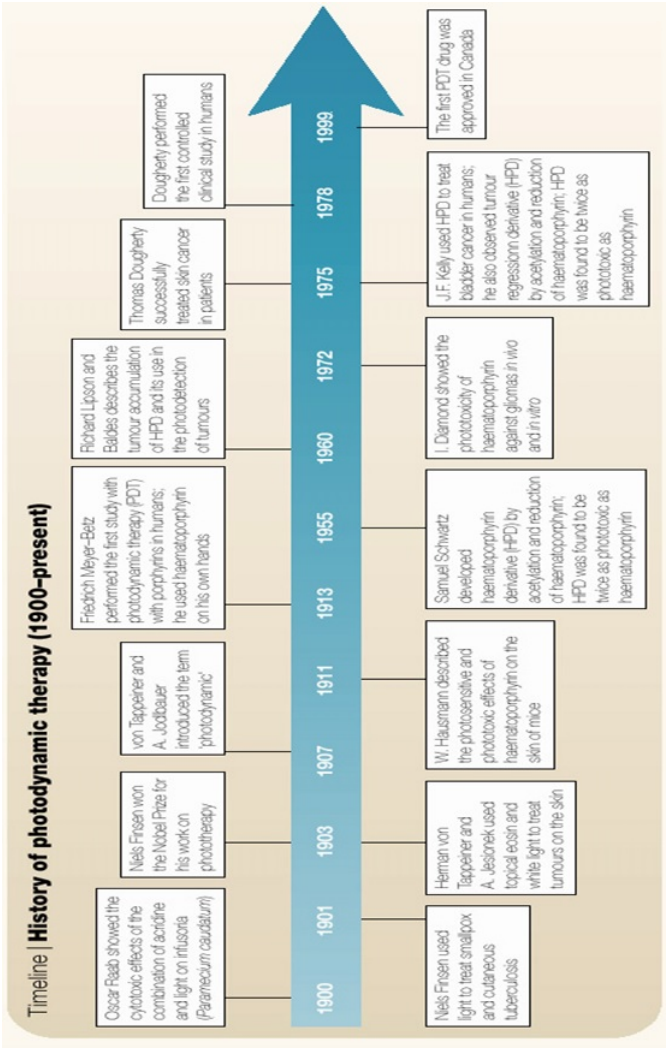

Figure 2.1 History of PDT between (1900-2000) [37].

diseases such as lupus, and arthritis etc. was published. The tumor selectivity of porphyrins using porphyrin fluorescence with systematic applications in tumor tissue of rats was examined in 1942 by Auler and Banzer. In 1955, Samuel Schwartz explored hematoporphyrin as a phototoxin and found a hematoporphyrin derivative (HPD) via the reduction of hematoporphyrin and acetylation. This was an important discovery because HPD has better photosensiting and localizing properties than other hematoporphyrins and porphyrins [16].

In 1960, Lipson and Baldes at the Mayo Clinic, injected hematoporphyrin in neoplastic cancerous tissues in rats and caused preferential fluorescence of these tu-

April 19, 1993	Canadian Health Protection Branch approved Photofrin or PDT
April 11, 1994	Axcan Pharma regulatory vast approved in the Netherlands for lung cancer.
October 5, 1994	Axcan Pharma regulatory vast approved in Japan for gastric cancer.
July 13, 1995	Axcan Pharma regulatory vast approved in USA for palliation of esophageal cancer.
April 9, 1996	Axcan Pharma regulatory vast approved in France for esophageal cancer.
July 30, 1997	Axcan Pharma regulatory vast approved in Germany for early stage lung cancer.
January 9, 1998	Axcan Pharma regulatory vast approved by US FDA for early stage lung cancer.
December 22, 1998	Axcan Pharma regulatory vast approved by US FDA for late stage lung cancer.
December 22, 1998	Axcan Pharma regulatory vast approved in UK for advanced lung cancer.
February 15, 1999	Axcan Pharma regulatory vast approved in Finland for advanced lung cancer.

Table 2.1
Current Status of product development [36].

mors. After this discovery, the researchers designed new photosensitizers for targeted tumors. In 1961, after Lipson's successful study about the usage of a combination of suitable doses of light and HpD photosensitizer as a treatment for women's breast cancer cells, PDT began to be used as a cancer therapy method. Following this study, PDT was used succesfully as a cancer therapy for skin tumors by Dougherty and bladder tumors by Kelly in 1975. In 1972, Diamond showed the light activation effect of HPD in gliomas of rats in vivo and in vitro. The results showed that HPD had a significant effect on both in vitro and vivo studies against gliomas. After the study, the new experiments were conducted using different HPD preparations during the mid

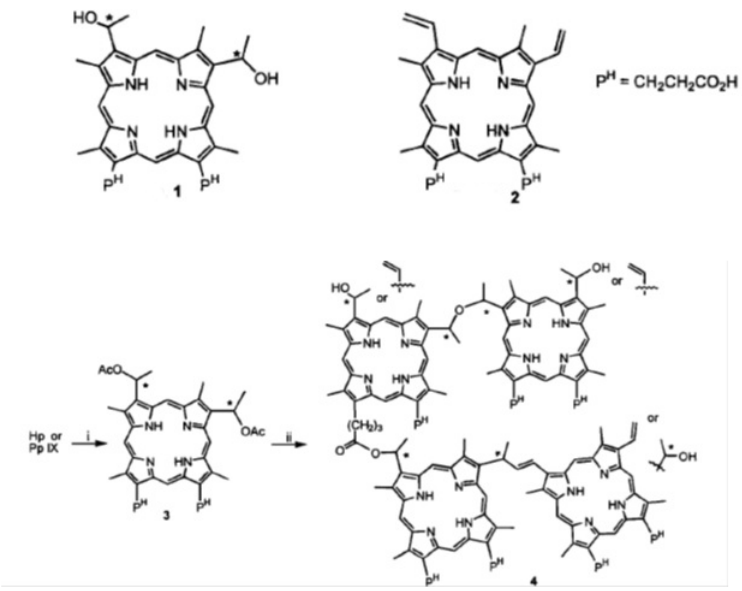

Figure 2.2 The History of the First Generation PDT Agent Hematoporphyrin Derivative 1. Hematoporphyrin (Hp) 2. Protoporphyrin IX (Pp IX) 3. Forming of Hp with sulfuric acid in acetic acid 4. (HpD) hematoporphyrin derivative. In this figure i. shows H_2SO_4, HOAc ,ii. shows 0.1 N NaOH as reaction conditions [33].

1970s. Dougherty did the first controlled clinical trial on the human body in 1978 [29],[33], [34],[35],[36],[38]. Since then several clinical studies of PDT in humans were done, also various photosensitizers are used on different cancer types such as breast cancer, gynaecological tumors, breast cancer, head and neck tumors, brain tumors, brain tumors and rectal cancer. J.S. McCaughan also used PDT for the treatment of oesophageal cancer in 1984 [34], [16].

The U.S. Food and Drug Administration (FDA) officially approved the purified HPD Photofrin (porfimer sodium) as the treatment for bladder cancer, in 1993 [32], [33]. Since the first official approval for PDT treatment of bladder cancer, many other health agencies have followed by approving PDT as treatment for other cancer types and non-oncologic cases until now. Between 1995-1999, PDT was approved for lung cancer and esophageal cancer in many countries throughout the world including Japan, Finland, and Canada by FDA license [36].

Currently many clinical studies have being done to localize photosensitizer effectively in PDT treatment. Since then, PDT applications have been extended into non-oncologic cases such as age-related macular degeneration. In 2000, the PDT agent δ-aminolevulenic (ALA) was approved as a treatment for actinic keratoses in the US by the FDA. Since then, ALA has been used as PDT agent for both malignant and non-malignant conditions all around the world. In 2001, another photosensitizer mTHPC was approved for the treatment of neck and head cancer in the European Union. In 2003, Photofrin in PDT was approved as an early stage treatment of Barret's esophagus which can cause esophegal adenocarcinoma [36], [16].

Increasing the PDT efficiency based on the importance of selectivity of photosensitizers and mechanism of tumor selectivity. Understanding of delivery systems in PDT and the structure of carrier moleculars such delivery systems of lysosome have an important role in PDT applications on efficiency of PDT photosensitizers [39], [40]. Since early 2000s, the usage of PDT in dermatology has an important development in bacterial and fungal skin infections. Antibacterial PDT has been considering as an alternative and effective treatment of several infections by fungi, bacteria abd viruses all around the world [32].

In recent years, due to the efficiency of antibacterial photodynamic theraphy, the usage of Photodynamic antimicrabial chemotherapy has been increased as an alternative treatment against fungal, bacterial and viral organisms [41]. Besides this, due to successful applications of PDT in cancer cases, in oncological problems, photodynamic chemotherapy has been represented as a promised new treatment.

Antibacterial photodynamic therapy represents safe and trustful treatment against multi-drug resistance bacteria due to its minimizing property of the bacterial resistance occurence and application in the targeted tissue such as wounds with suitable photo-

sensitizer [42].

2.1.2 Mechanisms of PDT

The cell killing mechanism of photodynamic therapy is based on combination of appropriate light energy, photosensitizer which has a molecule that absorbs the light energy and the presence of molecular oxygen which causes killing effect to the selective cell or the target tissue [1], [2], [3], [4].

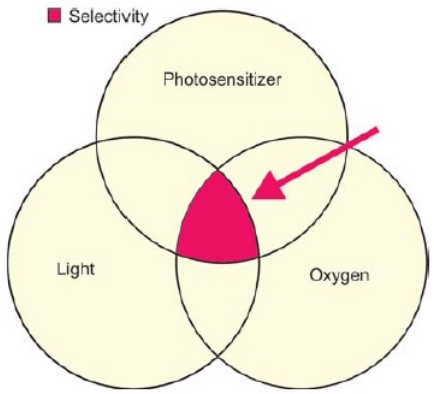

Figure 2.3 The selectivity factors of PDT [43].

Reactive free oxygen radicals are formed when the light energy at a specific wavelength activates the photosentsitizer that causes oxidizing membrane proteins of target cells. It leads to cell death via direct damage to membrane components or formation of reactive oxygen species [1], [2], [3], [4].

When photosensitizer is activated by the absorption light, energy is transferred from ground state to singlet-excited state (Figure 2.5). The killing mechanisms of PDT based on two types reactions in these oxygen molecules: Type I and Type II. The contributions of Type 1 and Type II mechanisms depend on a few factors such as the presence of oxygen molecules, the subsrate, the subcellular localization and the

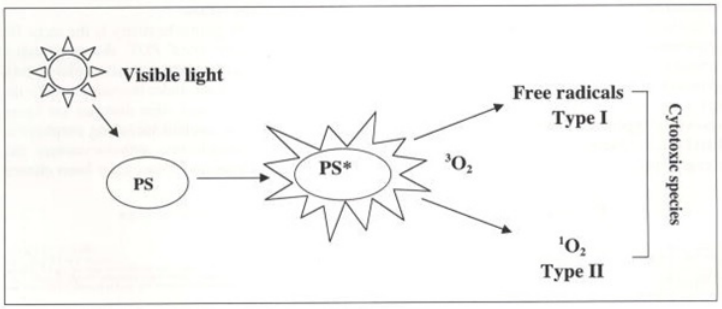

Figure 2.4 ROS effects on PDT [44].

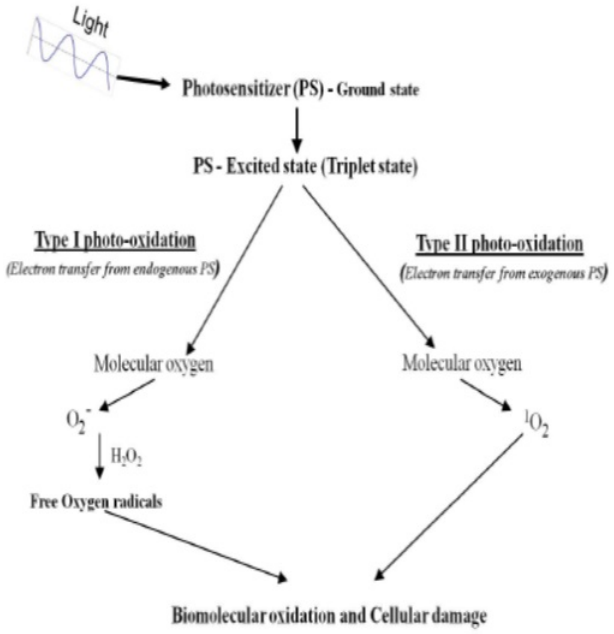

Figure 2.5 Schematic illustraion of PDT mechanism [45].

phososensitizer type. Activated photosensitizer via absorption of light causes energy transfer from ground state to singlet-excited state and to the triplet state level, respectively. In Type I, there is an interaction between electrons and neighbouring molecules with the triplet excited state and this causes formation of free oxygen radicals. For example , hydrogen peroxide and hypochlorite radicals are free oxygen radicals. Free oxygen radicals oxidize membrane protein. They are effective at killing the target cells. In Type II, singlet oxygen molecules are obtained by the energy transfer from triplet state photosensitzer to ground state moleculer oxygen. This oxygen molecules react with several biological molecules [44], [45],[46].

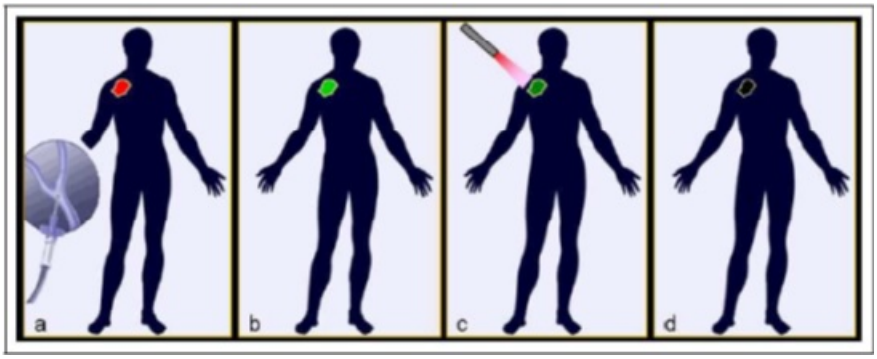

Figure 2.6 Mechanism of PDT in tumor cells killing [46].

The procedure of PDT in tumor applications is shown in Figure 2.6. First of all, the photosensitizer is injected to the target cells systemically or topically (a), then after waiting for a sufficient time, the retention of photosensitizer into the target cells are done (b), Following the first two steps, the light energy at a certain wavelength (laser irradiation in general) is applied to the targeted cells to produce reactive oxygen species (ROS) (c),the targeted cells are killed by ROS and recovery is occured (d) [47], [46].

2.1.3 Clinical Uses of PDT

Photodynamic therapy (PDT) has been used in several clinical applications since the treatment and various photosensitizing drugs has been approved by the FDA. New PDT techniques and clinical new developments of PDT increased attention on PDT in other uses beside malignant diseases [48].

Applications of PDT:

(a) Cancer Therapy

(b) PDT in Dermatology

(c) Antibacterial PDT

(d) Other Uses

(a) **Cancer Therapy**

- Barrett's esophagus
- Bladder cancer
- Brain cancer
- Eye cancer
- Esophageal cancer
- Lung cancer
- Head and neck Cancer
- Breast cancer
- Gynecologic malignancies
- Prostate cancer

PDT has been used as an effective treatment way for the above cancer types. It was approved especially as a clinical application by FDA.In recent studies, PDT is discovered as effective treatment way for localized prostate tumors. Padoporfin-mediated PDT, temeporfin-mediated PDT were successfully reported in some clinical studies [49], [50]. In Japan, Canada, USA and several European countries PDT with photofrin is approved as treatment for advanced lung cancer and early stage lung cancer. Although PDT for bladder cancer as treatment is approved in some European countries and in Canada by the FDA, it has not been approved in the USA [51],[52],[53],[51].

(b) **PDT in Dermatology**

- Actinic Keratoses

- Psoriasis Vulgaris
- Bowen's Disease
- Acne Vulgaris
- Basal Cell Carcinoma
- Scleroderma
- Lichen Planus

The main purpose of PDT uses in dermatology is the treatment of precursors of non-melanoma skin cancers. All the above dermatologic cases have been treated with PDT in the published literature [48].

(c) **Antibacterial PDT**

- Viral infections such as Herpes keratitis and human papilloma virus (HPV), blood products and warts etc.
- Bacterial infections such as Infected Wounds and oral cavity, yeast and fungal infections
- Dental infections
- Gastric infection
- Infections in brain abscesses

Although PDT was discovered to kill microorganisms with the presence of ROS and combination of light and suitable chemical dye many years ago, PDT has been started to be used as an alternative method to treat of various localized infections in the recent years. Especially, antimicrobial PDT has been searching in clinical applications against antibiotic resistant bacteria. Antibacterial treatment is effective and well-known treatment recently in dermatology and dentistry (in particular among wound infections, endodontic and periodontic infections) to kill multidrug resistant bacteria that cause infections [53], [54].

(d) **Other Uses**

- Hair removal

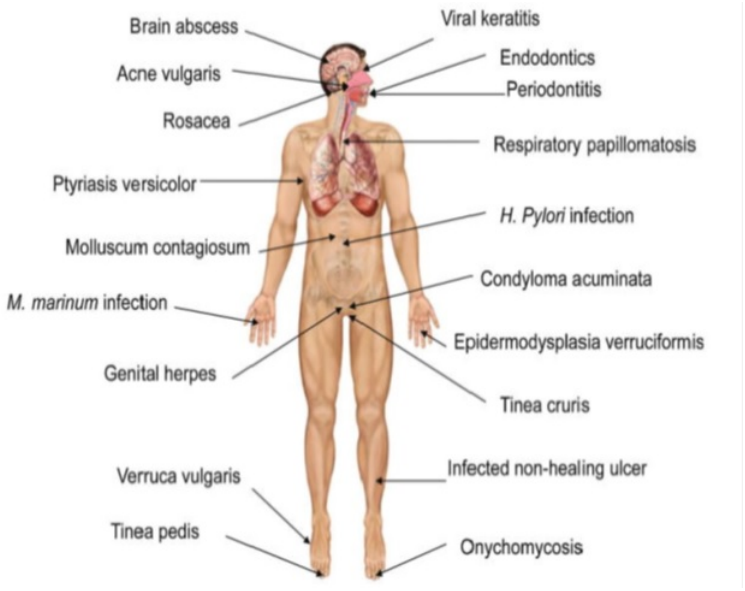

Figure 2.7 The scheme of clinical PDT applications on human body against infections [54].

- Age-Related Macular Degeneration
- Immunologic and Inflammatory Disorders
- Varicose Veins
- Verrucous hyperplasia
- Verrucous hyperplasia
- Oral Verrucous Hyperplasia
- Cardiovascular (Reduction of atherosclerosis lesion severity and stabilization of vulnerable plaque)
- Osteomyelitis

Photodynamic therapy (PDT) offers many advantages to treat cardiovascular diseases and vascular disorders. PDT shows a promising approach in cardiac and vascular surgery such as the inhibition of intimal hyperplasia. Free oxygen radicals in PDT can decrease cell migration by prompting apoptosis of the smooth muscle cells. Whereas several factors can cause vascular lesions during surgery, PDT can be safer treatment for vascular diseases. Since only target cells are being affected in PDT and surgeon is limited in intimal hyperplasia, PDT shows more promising way to overcome vascular disorders [48].

Recently, PDT has been used in treatment of age-related macular degeneration and immunologic disorders. Due to the interaction of PDT within cells, it influence on various immune parametres and can be efficacy against immunologic disorders. Besides, penetration of PDT deeper tissue and impacts on only target cells makes PDT better among treatment methods [48], [54], [55], [56].

PDT has been used in several clinical applications due to its advantages. These advantages are shown in table as follow:

	Advantages	Limitations	
		Fundamental	**Current Technology**
General	Platform technology with wide spectrum of potential applications.	Limited light penetration in tissues	No general purpose light systems
	Minimally invasive	(Hence) not generally applicable to systemic disease	Limited use and accuracy of patient-specific dosimetry and consequent treatment optimization
	Low systemic toxicity		
	Multiple mechanism of biological effect	Complex to optimize because of multiple factors	
	Can be repeated without inducing significant resistance or hypersensitivity		
	Highly 'portable' and relatively low-cost technologies		
Solid Tumors	Rapid effect in single treatment	Oxygen dependent (for one-photon excitation)	Limited target specificity of clinical photosensitizers
	Can be curative, palliative, or prevent progression	Challenging to achieve adequate treatment throughout larger solid tumor masses	
Age-related Macular Degenaration	Stops or slows disease progression	One-photon excitation may cause collateral damage	Needs to be repeated several times to halt progression
Localized Infection	Effective against a wide range of mircoorganisms, including antibiotic-resistant strains	Not known	Cost of light sources
	Topically applicable without inducing systematic toxicity or disease resistance		Delivery of photosensitizer over extended period

Figure 2.8 The main advantages and limitations of PDT in clinical applications [21].

2.1.4 Antibacterial PDT

Antibacterial photodynamic therapy has been considered as a remarkable alternative therapy for localized infections in recent years. The increaing interest for Antibacterial PDT has revived because of "the antibiotic era". A large variety of microbial infections caused by micro-organisms such as wound infections, burn infections, soft tissue infections, oral-dental infections and virus infections have been known for many centuries. The widespread use of antibiotics have been implemented to treat bacterial infections. But there is an urgent need to combat with bacterial infections treatment since many classes of pathogens and bacteria has been driving drug resistance and antibiotic resistance. This antibiotic resistance among bacteria emerged the antibiotic era and drove new research of effective strategies to treat the diseases caused by bacteria [1], [4],[10], [23], [57].

Antibacterial PDT is based on the combination of a dye (known as a photosensitizer), suitable doses of light at an appropriate wavelength (laser source is used in general) and presence of oxygen (free oxygen radicals or singlet oxygen molecules). In PDT application, the photosensitizer which is the suitable one in effective dose is localized in the bacteria to be activated by applied light energy at appropriate wavelength in order to generate singlet oxygen or free oxygen radicals to kill the targeted bacteria. Especially photosensitizer should be applied in bacteria, not the around tissue or cells. So the target cells are killed exclusively by singlet oxygen molecules or free oxygen radical that are produced via light absorbing photosentsitizer [10]. Antibacterial photodynamic process proceed by 2 pathways, Type1 and Type2, both of which require the presence of photo-excited triplet state of photosensitizer (PS*) as the reaction of light absortion.

Light activated the photosensitizer from ground state (PS) to the triplet (PS*), then in Type 1,free oxygen radicals are produced such as hydrogen peroxide, hydroxyl radicals and in Type 2 formation of reactive oxygen species (ROS) are alternated. In

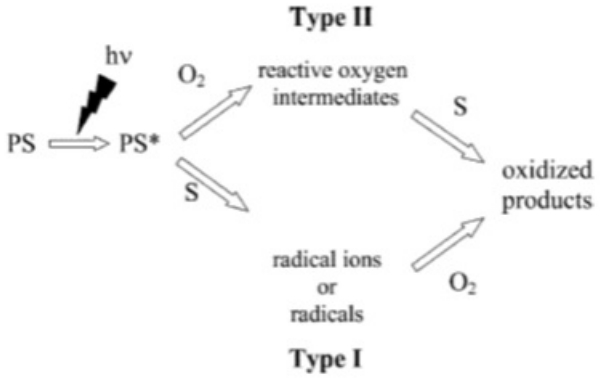

Figure 2.9 The basic mechanism of antibacterial PDT [10].

Type 1, an electron transfer between a nearby organic substrate (S) and the triplet photosensitizer is done by their relative redox potential. In Type 2 mechanism, oxidized product which is named singlet oxygen ($^1O_2$) is obtained thanks to energy transfer from the triplet photosensitizer, which are able to kill cells. Becuase this process turns into advantage of reacting with photosensitive targets in its surroundings such as cell wall, nucleic acids, cell membranes, proteins, blood. By this processes, antibacterial PDT play an important role to kill microbial cells effectively [1], [4],[10], [23], [57]. The photochemical processes of Type 1 and Type 2 produces ROS from phosensitizer triplet state to kill all known microorganisms which summarized in below figure [58].

There is a large scale of localized infections treated by Antibacterial PDT. After the wide various of multidrug resistance bacteria problem throught the world, antibacterial photodynamic therapy has been used increasingly in clinics as a common treatment for wound infections, burn infections, soft tissue infctions, oral-dental infections and virus infections. The summary of Antibacterial PDT used in clinically treatments is shown in below figure.

There are several advantages of Antibacterial PDT compare to Antibiotics. We can summarize them as follows:

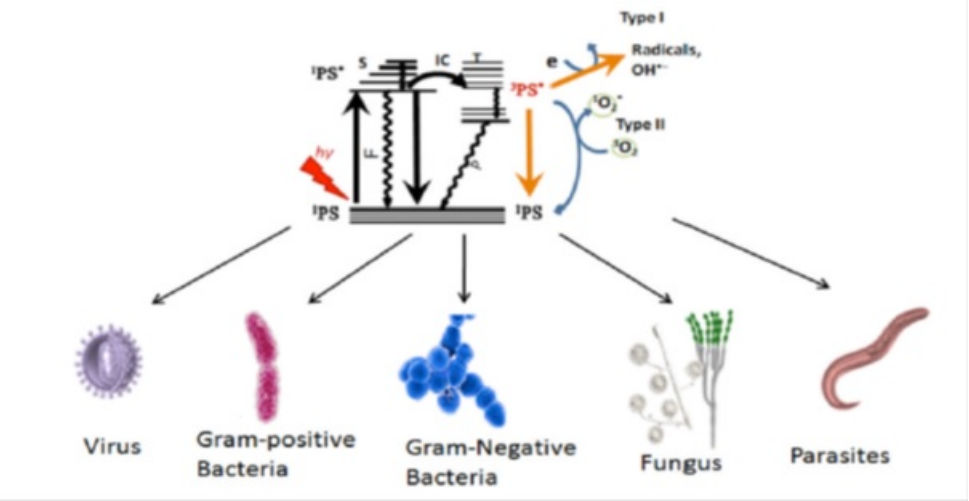

Figure 2.10 The shemactic mechanism of antimicrobial PDT to destroy all known microorganisms [58].

- Antibacterial PDT is safe on human health. It has limited severe effect on surrounding cells.
- After PDT application, bacteria do not develop resistance like they do against antibiotics.
- PDT is effective for killing bacteria and the other microorganisms fungi, and viruses.
- PDT application is shorter than antibiotic taking process.
- PDT eradicates pathogens in biofilms also.
- PDT has few side effects.
- Broad spectrum of action is larger since photosensitizer act on bacteria directly.
- Use of it is cheap. Suitable exposure wavelength of light sources for photosenstizer activation.
- Available process to eradicate infected area.
- It can be re-apply without causing photoresistant on bacteria [58],[59],[8],[60].

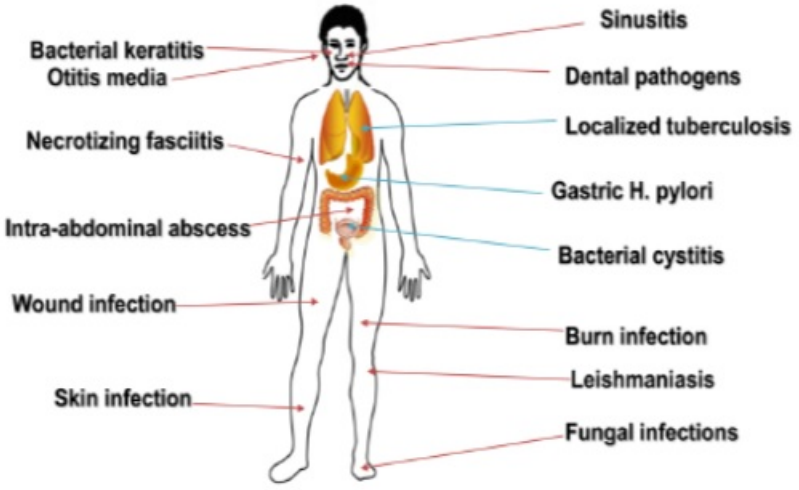

Figure 2.11 Clinical uses of Antibacterial PDT [1].

In the below figure the advantages of antibacterial PDT over antibiotics are shown.

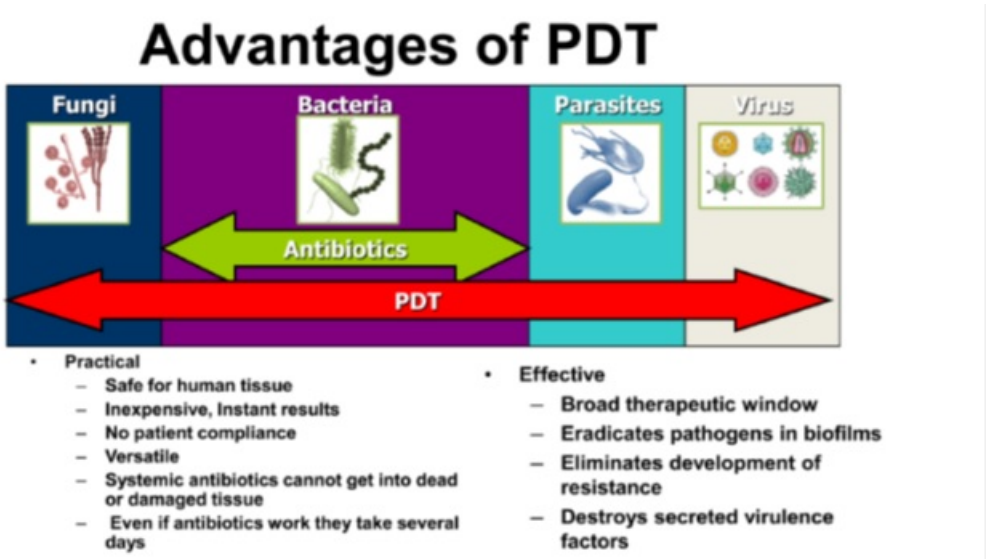

Figure 2.12 Advantages of antibacterial PDT [58].

While many antibiotics have narrow spectrum, antimicrobial PDT has larger spectrum on absorption band because the potential of photosensiters in PDT is larger and the absorption wavelength of light sources with photosensitizers is various like (NIR, visible light) [58]. Besides that, phosensitizer structure in Antibacterial PDT is

important because of interaction the surface charges of bacteria and the photosensitizer charge. For these reasons, the effect of Antibacterial PDT is rapid and more efficacy over antibiotics [58], [61].

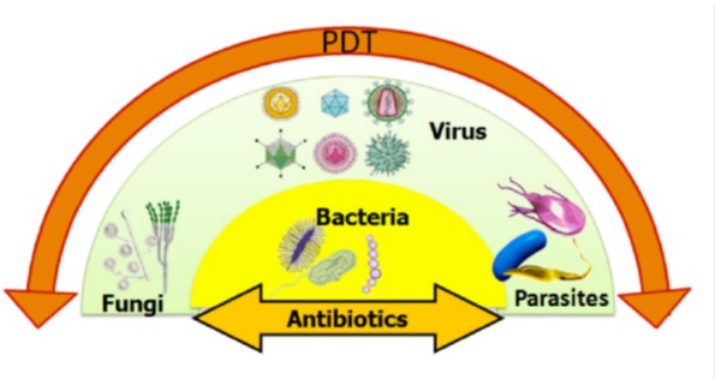

Figure 2.13 Effect of antimicrobial PDT on the broad spectrum [58].

2.1.5 PDT Dosimetry

Photodynamic therapy is a promising treatment for target cancer cells and multi-drug resistant bacterial infections. The main success of PDT for selectivity and efficacy is dependent on PDT dosimetry which is described within four parameters: light dosage, photosensitizer concentration dose and oxygen concentration and PDT time interval (duration between light irradiation and photosensitizer addition). Each of these parameters is an important factor to determine efficacy at each point in a target tissue or in target cell during PDT process [61],[62],[63].

Cell death response to PDT application is various dependent on biological environment type of target cell, physical properties, PDT dose, the photoactivation time. The efficacy of photosensitizer concentration is different from one person to another and varies throughout the body as a function of time. In addition to this, the penetration of light energy into the target cells is dependent on the specific optical-physical properties. It is believed that singlet oxygen plays an important role in the efficacy of PDT [4], [64],[65],[66],[67]. So the tissue type has an impact on the yield of produced

singlet oxygen by PDT. For example, when PDT dose is applied more than the limited one, little damage to surrounding normal tissue have observed or inadequate biological response to the treatment of unacceptable complications can be led by less PDT dose applications to the target cells such as biostimulation effect or still-living tumor response [68],[69],[70]. Light penetration, tissue oxygenation and the sensitizer concentration can change and affect each other during the photoactivation process (The amount of activated photosensitizer and ROS production). For these reasons, PDT dosimetry must be applied at the optimum doses in each parameter to get successful results [65],[70].

Reactive oxygen species (ROS) produced in PDT process play an important role in destroying bacteria via stimulating various biochemical pathways in the cells causing cell proliferation [65], [71],[72],[73]. So the oxygen radicals will be produced lower, if the drug and light dose is applied lower; and it can lead to undesired PDT results such as cell proliferation on bacteria and inhibitory effect cells causing cancer cells instead of PDT killing effect [74],[75].

All of the above parameters are essential for the proper evaluation of PDT dosimetry. They can be explained in details as the light source including wavelength, light dose, energy fluence rate, duration of irradiation and photosensitizer including type, concentration dose, route and administered dose, the patient's biological knowledge and PDT applied biological field of the body are the important parameters in PDT and they should be checked by nurses/physicians during PDT [61],[62],[63],[69],[70].

2.1.6 PDT with 809-nm Diode Laser and Indocyanine Green

Indocyanine green (ICG) is a water-soluble, anionic tricarbocyanine non-toxic dye [76],[77],[78],[79].The negatively charged molecule of ICG is shown as $C_{43}H_{47}N_2NaO_6S_2$

in organic chemistry [6].

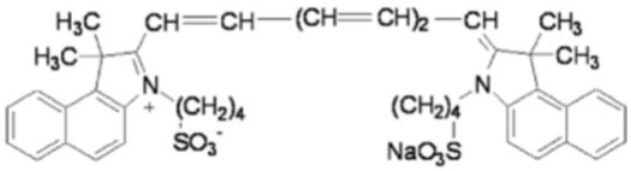

Figure 2.14 Chemical structure of ICG [76].

ICG has an absorption spectrum between 600-nm and 800-nm and its maximum absorbance is at wavelengths around 800-nm [80],[81].

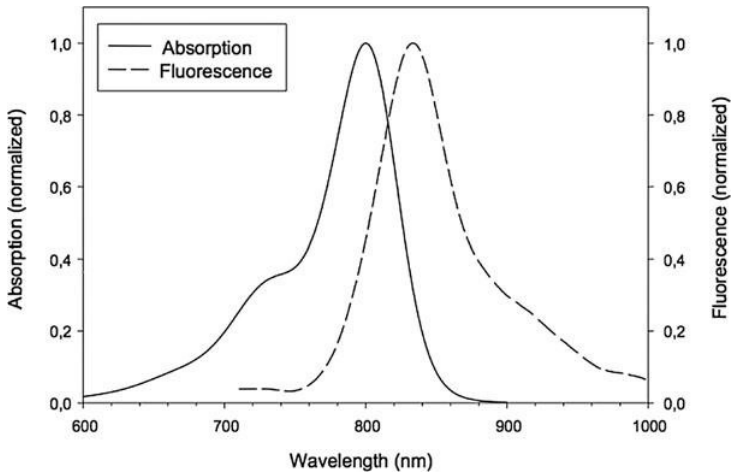

Figure 2.15 Absorption and fluorescence spectra of indocyanine green (ICG). Documents from Pulsion Medical System [77].

Indocyanine green (ICG) has been approved by the United States Food and Drug Administration (US FDA) for medical applicatios such as a dignastic tool in hemodynamics [19], a contrast agent in medical imaging for cardiac output, blood volume [77], [78].

ICG is an important photosensitizer in photodynamic treatments with diode laser application due to being cheap and efficient. ICG exhibits a maximum penetration of light into the deeper tissues in the Near infrared spectral region without causing

significant heating (around 800-nm). Especially diode laser usage is common as the near infrared light source with ICG in PDT clinical applications to treat deeper tumors such as skin cancer, human keratinocytes, breast cancer and human plasma problems. Moreover there is some research of PDT usage to treat Choroidal neovascularization with Age-related macular degeneration via ICG-diode laser application [77], [82], [83]. Diode laser has a greater capacity to penetrate deeper tissue when ICG is applied [76], [80].

Diode lasers are very trustable and efficient, and their usage is very common in medical applications due to high clinical safety. PDT and fluorescense diagnostics are application fields of diode lasers [84].

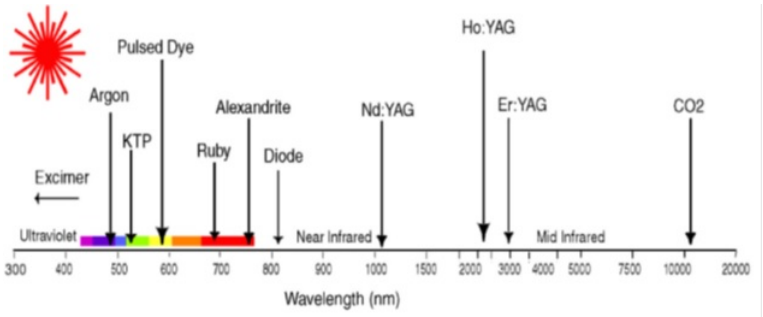

Figure 2.16 Diode lasers and other medical lasers [84].

Some researches show that PDT with ICG and diode laser irradiation has been using for acne treatment [77], [85]. Recently, PDT with ICG via diode laser irradiation has been investigated to see the effect on antibiotic resistant bacteria [77], [80].

In our study, we used Indocyanine green and 809-nm diode laser combination on wild type P.aeruginosa which cause to wound infections. Our data resulsts show that ICG and 809-nm diode laser combination can be a strong way to combat the ongoing antibiotic resistant bacteria problem all around the world.

2.2 Wound Infecting and Wound Infections

2.2.1 Gram+,Gram-Bacteria

Infected wounds lead to morbidity and mortality which is a considerable problem throughout the world due to drug resistance bacteria. Current antimicrobial treatments have not been as effective so far because of increased antibiotic resistant bacteria in infectious diseases such as burn wounds [1], [54]. On the other hand, PDT has some promises for the treatment of localized infection. The reactive oxygen species produced by PDT reduce the effect and the level of bacteria on human health [4], [10] .

Microbial cells are categorized by large differences based on their cellular structure and organization. However, phosensitizers are effective toward all wild strain bacteria, antibitiotic restistant gram-positive bacteria (for example: Staphylococcus aureus) and gram-negative bacteria (for example: Pseudomonas aeruginosa). The photosensitized process of PDT is applied differently to gram-positive bacteria and to gram-negative bacteria because the thickness of outer wall of these bacteria are different from each other and it leads to different efficiency of photosensitizers with cell constituents. For this reason, PDT dose and mechanism of the photoinactivation process of antimicrobial PDT having an important role to combat with these bacteria. The photodynamic process activity on such cells, in the cytoplasmic membrane is the prerequisite for main target. Since the combination of the outer wall and photosensitizer has the key role to destroy microbial cells which show a large variety in size, biochemical composition and susceptibility to phosensitized process [8].

Gram-positive bacteria are surrounded by an outer wall, which is seperated from the plasma membrane by 15-80-nm thick wall. The outer wall of gram-positive bacteria has porous structure and includes up to 100 peptidoglycan layers which are related to negatively charged teichuronic acids and lipoteichoic acids. On the other hand, gram-negative bacteria are characterized by the presence of additional 10-15-nm thick

structural element, comprosing of lipoproteins and lipopolysaccharide trimers, which makes it a very heterogeneus structure and external layer to network occured of peptidoglycan [8],[60], [54]. The non-porous cell wall structure in gram-negative bacteria includes in the outer membrane and inner cytoplasmic membrane and peptidoglycan-containing periplasm seperates them from each other [58].

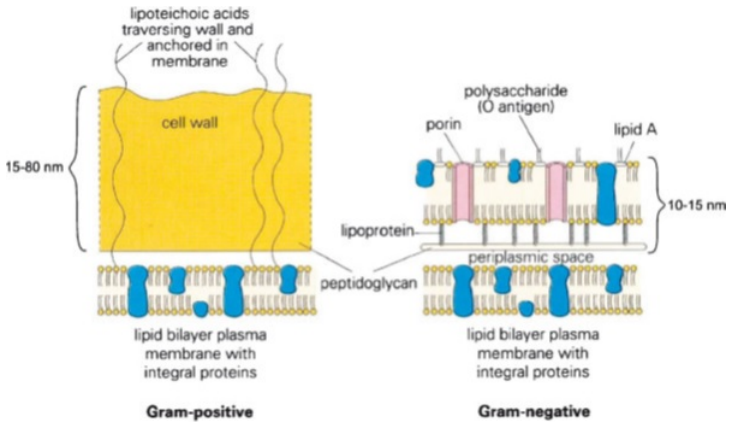

Figure 2.17 The outer wall and the cytoplasmic membrane structure in Gram-positive bacteria and Gram-negative bacteria [8].

The outer wall of gram-positive bacteria have a kind of barrier function due to its porosity structure and it isn't permeatable for the macromolecules over 1500-1800 Da. Because of this, the outer wall of gram-positive bacteria is not mostly permeatable barrier for the photosensitizer which are commonly used. The outer surface of gram-negative bacteria is negatively charged. And this mechanism shows resistant against several antibiotic drugs based on its well-organized system. It causes to allow only diffusion of relatively hydrolic compounds weighted lower than 600-700 Da through the porin channels. Thus, it inhibits the necessity of some suitable strategies to enhance the permeability of the outer wall of Gram-bacteria to increase the sensitivity to the photosensitizing action of photodynamic process in antimicrobial PDT [58], [8].

The differeces in susceptibility to antibacterial PDT between gram-positive bacteria and gram-negative bacteria is based on the accumulation in significant doses at the cytoplasmic membrane of available photosensitizers due to morphological differences

of gram-positive bacteria and gram-negative bacteria. This difference in susceptibility to antibacterial PDT between gram (+) and gram (-) bacteria has an important role to damage the bacteria. The recent studies show that antimicrobial PDT kills gram-positive bacteria by the use of anionic photosensitizers via binding efficiently to the outer wall of gram-positive bacteria. In this way, the photosensitizer exits the wall and destroys the bacteria. On the contrary, photosensitizers binding to the outer membrane of the gram-negative bacteria are not as efficient as the gram-positive bacteria's ones because of the presence of the barrier structure in the gram-negative bacteria. For this reason, gram-positive bacteria show sensitivity to the antimicrobial PDT remarkably while gram-negative ones show some resistance to the drugs. So, PDT dose has very critical role here to increase the photosensitizing action with the suitable photosensitizer absorbing light to destroy gram-negative bacteria [8], [54].

Besides gram-positive bacteria and gram-negative bacteria, fungal cells cause infections on human health. The permeability of outer walls of fungal cells is intermediate compared to gram-positive and gram-negative bacteria [58].

The structures of the cell walls of gram-postive bacteria, gram-negative bacteria and fungal cells are shown respectively below Figure. In summary, the outer wall of gram-postive bacteria includes in single lipid bilayer and peptidoglycan which shows porous layer. Gram-negative bacteria contains an outer layer and inner cytoplasmic membrane and this strucure shows a kind of barrier property. Fungal cells have less porous layer feature than gram-negative bacteria [58], [54].

The importance of susceptibility differences between Gram-positive and Gram-negative bacteria in Antibacterial PDT was discovered by the efficacy of neutral or anionic photosensitizer which could eradicate gram-positive bacteria, but not gram-negative bacteria. Because these PDT applications were including lipopolysaccharide permeabilizers whose usage was in concert with PDT process. After much research,

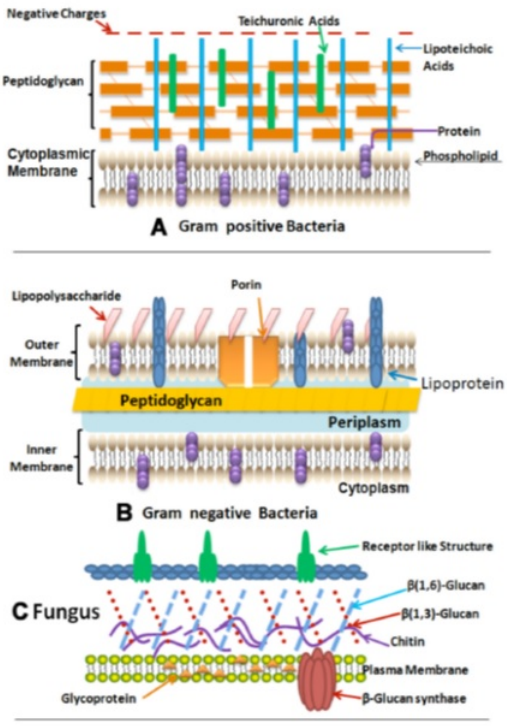

Figure 2.18 Cell structure of pathogens [58].

yielded cationic PS effect on both killing on gram-positive and gram-negative bacteria was proven, although both gram-negative and gram-positive bacteria have anionic outer cell structures. After the important discovery, antibacterial PDT has been consering as potential treatment for eradication of resistant pathogens [86].

2.2.2 Antibiotic Resistance

Antibiotic resistance is an urgent ongoing worldwide problem. Today, almost all mortal and risky infections caused by bacteria throughout the world are becoming resistant to antibiotics. This issue has been one of the biggest concerns for public human health. Antibiotic resistance can be described briefly as the ability of microorganisms including bacteria, viruses, fungi to resist the killing effect of antibiotics (they are also known as antimicrobial drugs) [87], [88].

Although antibiotics were very powerful drugs when they were used for treatment in 1920s, they are not effective cures for all infections any longer due to the increasing resistance of bacteria. Antibiotic usage in hospitals has been the key to combat infections caused by bacteria. But today, new forms of antibiotic resistance have spread to all countries as a big problem. Each year in countries all around the world over 2 million people have serious infections with bacteria which are resistant to at least one antibiotic [87], [88].

Antibiotics are the molecules that stop the growth of microorganisms or kill microorganisms (consisting of both bacteria and fungi). Those which kill bacteria are named "bactericidal" while "bacteriostatic" is the name of antibiotics which stop the growth of bacteria [89].

Infections	**Gram (-) Pathogens**	**Gram (+) Pathogens**
Burns	Pseudomonas aeruginosa	Staphylococcus auerus
Skin infections		S. aureus
Throat		Streptococcus pyogenes
Otitis media	Haemophilus influenzae	Streptococcus pneumoiae
Pneumonia	H. influenzae	S. pneumoiae
Endocarditis		S. aureus, Enterococcus faecalis
Septicemia	Escheria coli	S. aureus, S. pyogenes
Gastrointestinal tract	Salmonelaa enterica serovar, Thyphimurium, Helicobacter pylori, E. coli, Shigella dysenteria	
Urinary tract	E. coli	Enterococcus sp.

Table 2.2
Common infections and respective types of bacteria

The role of antibiotics to destroy bacteria shows one of the most important progresses made in scientific field which lead to the eradication of some incurable diseases in the early 21^{st} century. However, bacteria have developed resistance against many antibiotic mechanisms which were extremely effective before. In addition to this, bacteria replicate by developing resistance and insuring the survival of bacteria in the presence of antibiotic drugs, and, therefore, these resistant bacteria become the predominant ones among the microorganisms [60].

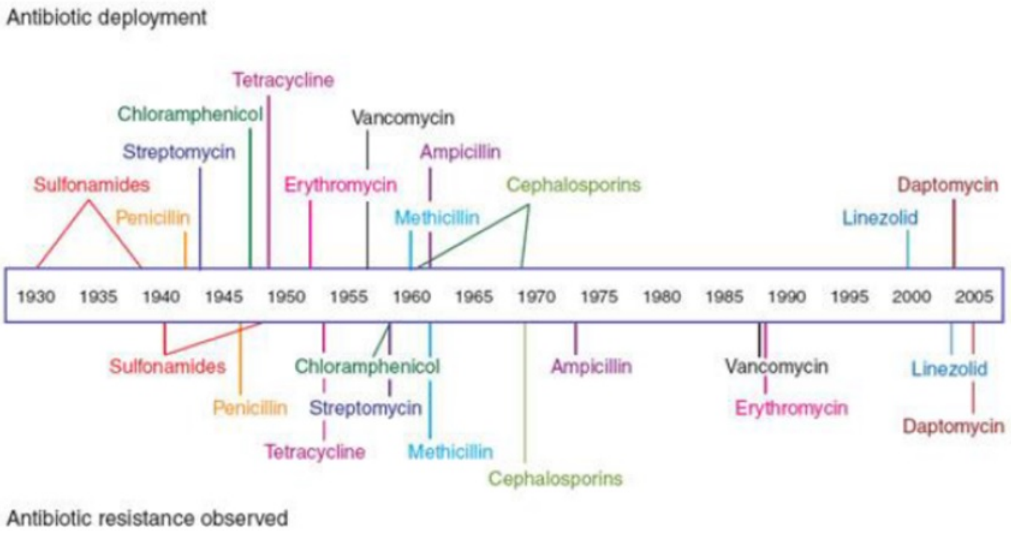

Figure 2.19 History of PDT between (1900-2000) [37].

To combat with bacterial infections, the understanding of the chemical nature and mechanisms of bacteria-drug interaction is a crucial way to find a solution how bacteria develop resistance against antibiotics [90] .

The mechanisms of action of antimicrobial agents are related to the effect of the biological structure of bacteria and the function of agents on bacteria can be categorized as the inhibition of :

- cell membrane function
- nucleic acid synthesis

- ribosome function
- cell wall synthesis

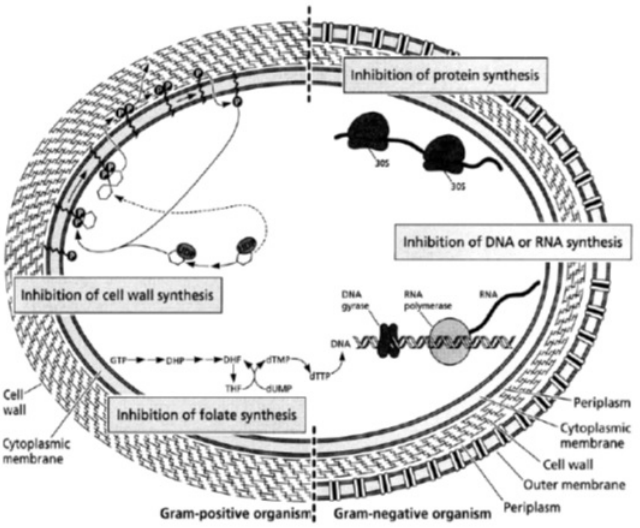

Figure 2.20 Mechanism of antibiotic action and resistance on both gram-positive and gram-negative bacteria [91].

Antibiotic resistance can be described as intrinsic resistance and acquired resistance:

- **Intrinsic Resistance**: It occurs naturally in all or most microorganisms and makes bacteria encoded chromosomally. In more details, it is desrcibed that microorganisms naturally are not affected from drugs due to the differences in the bacteria cell membrane structures and chemical nature of drug. Therefore, bacteria develops resistance against drugs via being encoded chromosomally.
- **Acquired Resistance**: It results from acquition of new DNA or the mutation in the existing DNA of a susceptible organism [90].

Antibacterial resistance causes not only failure of treatment but also spread of resistant germs. The problem of infections caused by resistant bacteria has no boundaries [90] . Especially, the three most dangerous bacteria that threaten the lives of

people all around the world are Enterococcus faecalis, Mycobacterium tuberculosis and Pseudomonas aeruginosa. They have resistance against over 100 drugs already [87]. Pseudomonas aeruginosa causes infections in wounds alarmingly due to the antibiotic resistant and makes scientist look for new treatment methods like antibacterial PDT [77].

2.2.3 Wound Infections

The clinical definition of wound infection has symptoms such as pain, erythema, edema, heat,and purulent according to correlation of bacteria quantity in a wound to infection. The role and significance of microorganisms in wound healing has been morbidity and mortality for many years. Bacteria has been known as a cause of serious wound and surgical infections types for many years. But the list of bacteria causing infections on skin and soft tissue infections is growing [92], [93],[94].

The presence of bacteria in a wound may lead in contamination, colonisation and infection.

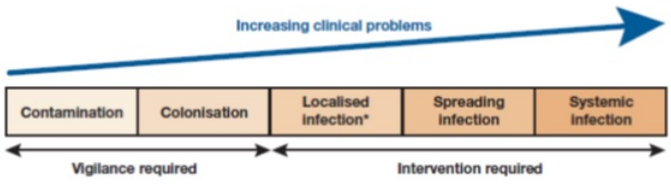

Figure 2.21 The presence types of bacteria in wound [94].

In contamination, bacteria neither cause clinical problems nor increase in number while bacteria increase in number but wound tissues are not damaged in colonosation. On the other hand, in infection issue, bacteria increase in number and cause damage in wounds and this situation may result in several problems like serious infection problems and altered pain [94].

Acute wounds and chronic wounds can be categorized under certain wound types. Acute wounds are caused by external damage such as surgical wounds, burns, minor cuts and bites etc. According to the depth and size of an acute wound is expected to heal within a predictable time. However, chronic wounds are associated with the integrity of epidermal -dermal tissue and endogenous mechanism. Leg ulcers, pressure sores and metabolic diseases such as diabetes mellitus are exampled as chronic wounds [95].

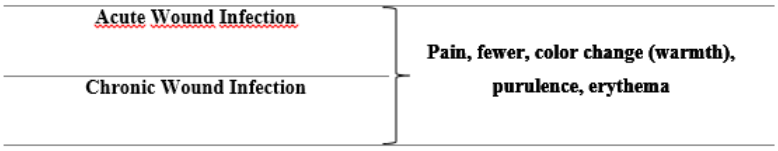

Figure 2.22 The common symptoms of both acute and chronic wound infection[96].

A need for new developed treatments for wound infections is clearly urgent to reduce patient morbidity. Especially burn inections and infected chronic wounds such diabetic infections are very dangerous due to incresing antibioic resistant bacteria. Bacteria are colonized in wound and made bacterial burden which leds to infection. It is very dangerous especially in local wound infections due to the resistance of bacteria. Because it cause delayed healing and serious drainage and pain along wound site in the infected wounds [96],[97].

In recent years, scientists and medical doctors are worried about surgical wounds which are under risk due to infection caused by pathogens. Because antimicrobial drugs in hospitals are used repeatedly result in becoming resistant bacteria to these antimicrobial drugs selectively and nosocomial infections are turning into ongoing morbidity problem. The below figure gives an idea about the importance of pathogens in hospital related infections [96].

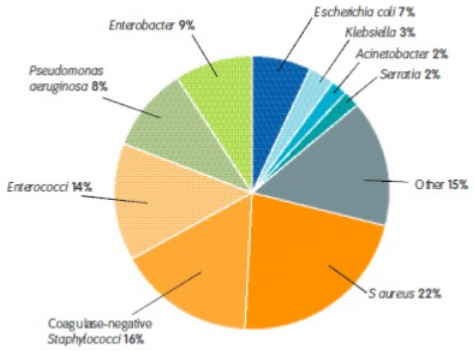

Figure 2.23 The bacteria in hospitals are a risk to cause infections on surgical wounds [96].

Pseudomanas aeruginosa is one of the most dangeorus bacteria which leads nonosocomial infections. P. Aeruginosa has high remarkable ability to acquire resistance against antibiotics. Moreover, acquired resistance of P. Aeruginosa increases by mutation or some mechanisms associated its cell structure and chemical agents applied on bacteria such as reduced permeability, degrading enzymes, active efflux and target modification. According to data, Pseudomanas aeruginosa is the third most common bacteria in intensive care units in hospitals and the second most common bacteria that cause nonosocomial infections in the urinary tract. In order to stop this urgent problem, antibacterial PDT applications on P.aeruginosa can be very efficient way to get rid of this problem as long as the suitable doses are applied to get PDT killing effect and avoid the risk of biostimulation effect on P.aeruginosa [77], [98],[99].

2.3 Biostimulative Effect

2.3.1 What Is Biostimulation?

Biostimulation is a kind of biochemical mechanism that stimulates cellular proliferation by low laser irradiation at cellular level in various ways including an increase in DNA synthesis and oxygen consumption, membrane potential, stimulation of cell

viability, and ATP production [100], [12].

'Laser Biostimulation' was coined for the first time in 1967 by Endre Mester, who was scientist at Semmelweis University, Budapest, Hungary. Low laser irradiation has an important role in obtaining a biostimulation effect via excitation of endogenous porphyrins which cause production of singlet oxygen and photoactivation of calcium channels led to increase in cellular proliferation [12].

Recent research indicates that mitochondria has remarkably effect in low laser light therapy (LLLT) mechanism. Because mitochondria is responsible for energy generation in cells, in addition to this the cellular response of mitochondria to NIR low energy results in ATP production by photon absorption by cytochromes in the mitochondrial respiratory chain affecting electron transfer.

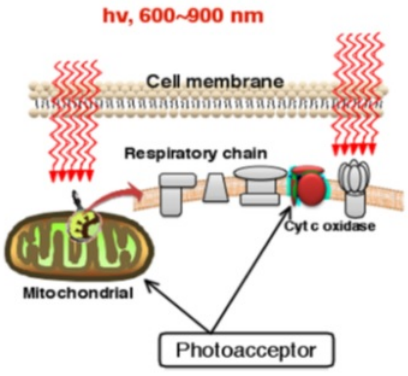

Figure 2.24 Schematic diagram of visible and NIR photon absorption of by cellular chromes in the mitochondrial respiratory chain [12],[101],[75],[102].

The studies of Hamblin indicates that the the production of singlet oxygen, a reactive oxygen species (ROS), (it is known as 'free oxygen radicals') obtained by photon absorption of low laser light application. Moreover, reactive oxygen species (ROS) took place from mitochondria to nuclei along the biochemical pathways such as cell growth and gene expression. ROS have an impact on cell proliferation too. At low concentrations of ROS, these biochemical pathways are stimulated by oxidative stress.

Lower concenration of ROS that led to oxidative stress may induce biostimulative effect on bacteria [26], [101], [21].

2.3.2 Low Light Source and Biostimulation

The use of low light sources (visible or near infrared light) has been used as a therapy for over 40 years. The first use of low level light therapy (LLLT) was reported in 1971 to heal wound. LLLT is an alternative method to treat for skeletal muscle generation, to heal wounds, and to enhance the proliferation of various cultured cells including stem cells since the development of lasers for medical uses. Low Level Laser/Light Therapy is known as laser biostimulation as well [12],[100],[101]. The stimulatory effects of low energy laser irradiation on cell activation have been shown in several vitro studies such as keratinocytes accelerate mitosis with application of low energy dose of diode laser [26].

LLLT for medical purposes has been used in a large scale. The use of low light sources is helpful for reducing pain and inflammation, healing wounds and deeper tissues, promotion of skeletal muscle regeneration etc [21],[103]. The low laser therapy has an important role on cell proliferation as well [103]. LLLT as laser biostimulation enhances the proliferation of some cells like cardiac stem cells and it modulates cellular metabolic processes as well. There are some examples of LLLT use in PDT for tumor treatment and some dermatological disease [103],[104].

Although there are several positive reports about low laser therapy, there are some negative ones too. The success of the applications is based on two reasons which are biochemical mechanisms and several parameters such as light energy dose, wavelength fluence, and treatment timing. Cell responses to the applied doses are better at low levels in particular. Mitochondria increases ATP production and production of

reactive oxygen species that leads to the increase cell proliferation and modulation in cytokines levels, cell growth factors and a rise tissue oxygenation [26], [100]. While these biochemical mechanism and cellular changes have several benefits on human health such healing wounds, amelioration of damage after heart attacks and retinal toxicity, it might cause biostimulative effect on bacteria cells instead of PDT killing effect or unwanted cells which can cause cancer tissue [75], [103], [105].

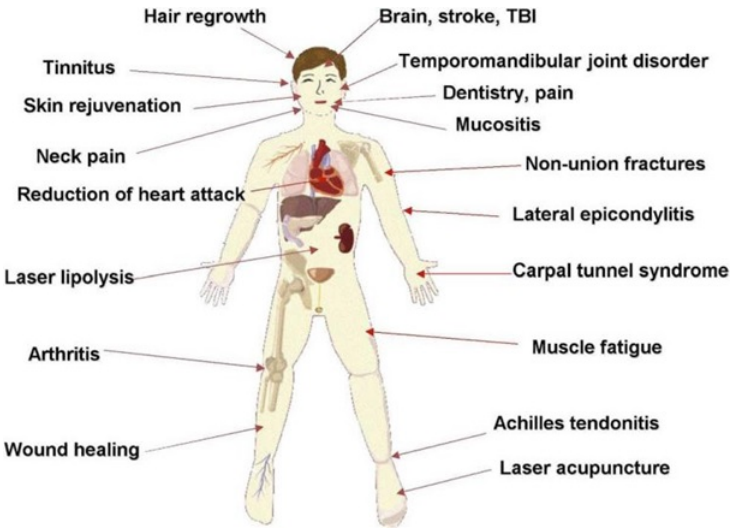

Figure 2.25 Diagram of the various medical applications of low-level light therapy [101].

Low Light Laser Therapy enhances regenerative processes of biological tisses with the laser biostimuation mechanism effect on tissue. These positive biostimulative effects on LLLT on tissues are shown in figure. For example : Hair regrowth, wound healing, reduction of heart attack, laser acupuncture etc. [100], [101].

2.3.3 Effect of Oxygen Radicals (H_2O_2,Free O_2 Radicals in PDT

Reactive oxygen species (ROS) have an important role in combating with bacteria or cancer cells in PDT. Most ROS are made by various redox mechanisms. There are 4 major ROS :

- Superoxide (O_2^-)
- Singlet oxygen ($^1O_2$)
- Hydrogen peroxide (H_2O_2)
- Hydroxyl radical (*OH)

These 4 major reactive oxygen species have very different kinetics and activity levels. The singlet oxygen molecules and hydroxyl radicals are more reactive then hydrogen peroxides and superoxides, and the less reactive ones are induced by oxidative stress. In photodynamic therapy, the presence of ROS is highlighted, because produced ROS during photoactivation process is responsible for killing the target cells in PDT. Recently, new approaches including ROS formation are popular as medical methods such as cell proliferation in stem cell regeneration and new antimicrobial defense like PDT [101], [106].

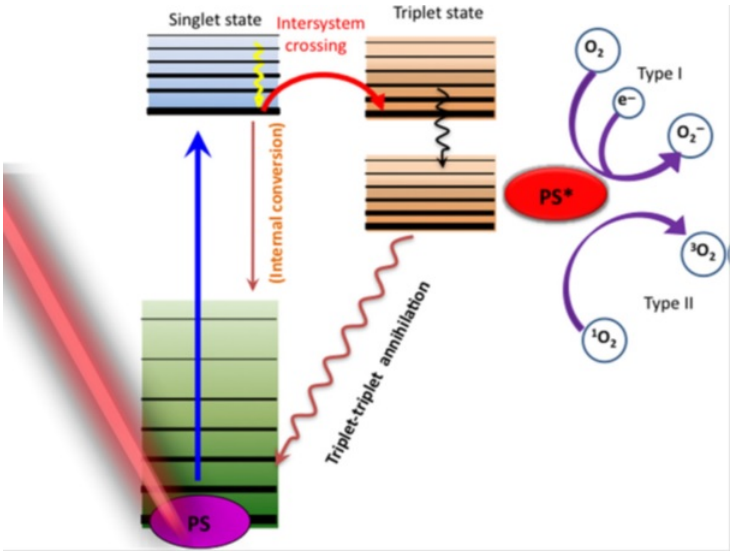

Figure 2.26 Schematic illustration of ROS production during PDT Type I and Type II mechanisms [106].

In PDT during the photoactivation process, photosensitizer after light irradiation moves from the ground state to the excited state. Excited state photosensitizer

transfers its energy to the ground triplet oxygen state leading photosensitizer's returning to the ground singlet state and the oxygen molecules moving to the excited singlet state (Type II). In Type I process, reactive oxygen species (ROS) such as singlet oxygen and free oxygen radicals which are highly cytotoxic that interact with lipids, proteins and nucleic acids in cells. These ROS are responsible for success of PDT process due to the reaction of cytotoxic reactions between ROS and cells (the details are explained in Chapter 2.1 under the title of Photodynamic Therapy) [44] .

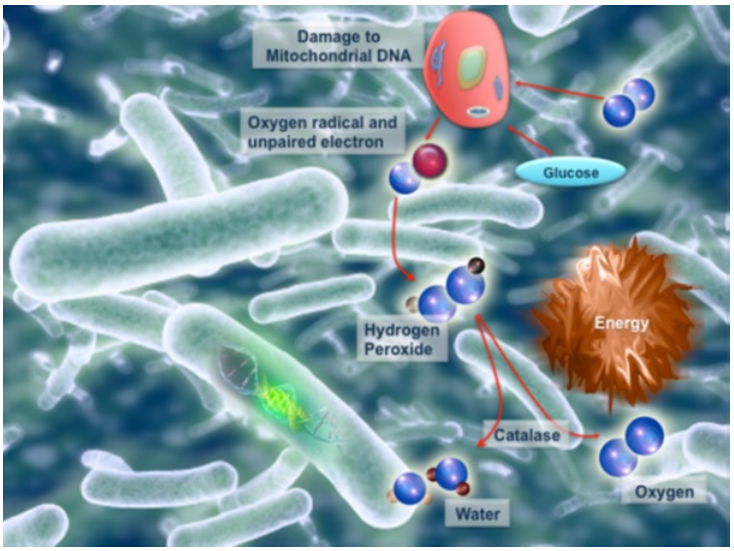

Figure 2.27 Production of ROS by superoxide leakage from mitochondrial respiratory chain. Hydrogen Peroxides and hydrogen radicals are formed by oxidative stress. The generation and interactions during ROS production from the mitochondria can damage host mammalian cells [106].

In eukaryotic cells, mitochondria is a kind of power house of the cell which is source of ROS. ROS have an important role on signaling, enzyme activation and cell cycle progression, protein synthesis, ATP production via biochemical pathways about from mitochondria to nuclei. Because ROS forms as oxygen metabolism by-products with interaction biological molecules in cells. ROS are very small molecules including ions such as superoxides and free radicals such as hydrogen peroxides and hydroxyl radicals which react with proteins, lipids etc. For these reasons, ROS are very important in Low Light Laser Therapy as well. Oxidative stress stimulates the biochemical pathways such as cell growth and gene expression, cell proliferation. For this reason, a lower amount of ROS is critical in antibacterial PDT and LLLT. It could lead to ox-

idative stress inducing biostimulative effect instead of killing effect of PDT on bacteria [4], [101], [106],[107].

3. MATERIALS AND METHODS

In this study, in order to observe the effects of ICG-PDT and to determine efficiency of PDT region, different energy densities with different ICG concentrations were applied on Pseudomonas aeruginosa (ATCC 27853) in vitro. In this study, the experiments were done in Molecular Biology and Genetics Department, Bogazici University.

3.1 Bacteria

In this study, in order to observe the effects of ICG-PDT and to determine efficiency of PDT region, different energy densities with different ICG concentrations were applied on Pseudomonas aeruginosa (ATCC 27853) *in vitro*. In this study, the experiments were done in Molecular Biology and Genetics Department, Bogazici University.

3.2 Photosensitizer

As a photosensitizer, indocyanine green (ICG) was used in this study. It was obtained from Pulsion, Medical Systems AG, Munchen, Germany. Fresh solutions of ICG were prepared in PBS at certain concentrations prior to each experiment and kept in the dark to protect from the light. In both laser groups and non-laser groups, ICG concentrations were assessed among range from 20 μg/ml to 250 μg/ml. ICG has peak spectral absorption around 800-nm wavelength.

3.3 Laser Light

In this experiments, computer controlled high power 809-nm diode laser (10 W output power at 35 A applied current) was used. The laser system was designed in Bogazici University, Biomedical Engineering Institute, Biophotonics Laboratory to use for irradiation to the target cells, tissue parts [82].

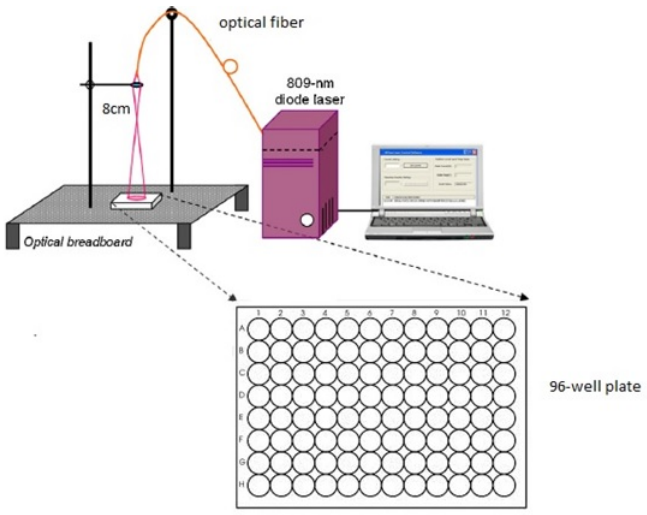

Figure 3.1 Laser System Set-up.

In each experiments, laser irradiation was applied with equal amount via predication on the laser probe distance to the plate surface. 84 J/cm^2, 168 J/cm^2 and 252 J/cm^2 energy doses were used in PDT and laser groups. The energy doses were applied by increasing the exposure duration in 60, 120 and 180 seconds respectively. In each experiment, laser power was checked with an optical powermeter which is obtained from Newport 1918-C, CA, USA. In each experiment, the optical fiber of laser system was set as 1.4 W/cm^2 to the plate surface.

Figure 3.2 Diode Laser System.

3.4 *In vitro* Studies

In this study, experimental groups were designed as Control group, PDT groups, ICG groups and Laser Groups.

- **Control Group**
- **PDT Groups** : In PDT groups PDT(xx/yy) was used to signify PDT laser doses (xx) and the photosentsitizer concentrations (yy) as [(J/cm^2)/(μg/mL)]. *For example:* PDT(84/20) shows that 84 J/cm^2 energy dose was applied with 20 μg/mL ICG concentrations in PDT experiment.

  PDT groups design: PDT(84/20), PDT(84/50), PDT(84/100), PDT(84/125), PDT(84/150), PDT(84/200) and PDT(84/250), PDT(168/20), PDT(168/50), PDT(168/100), PDT(168/125), PDT(168/150) and PDT(168/200), PDT(252/20), PDT(252/50), PDT(252/100), PDT(252/125) and PDT(252/150)
- **ICG Groups**: In ICG groups ICG(yy) was used to signify the photosentsitizer concentrations (yy) as (μg/mL). *For example:* ICG(20) means 20μg/mL ICG concentration.

  ICG groups design: ICG(20), ICG(50), ICG(100), ICG(125), ICG(150), ICG(200) and ICG(250)

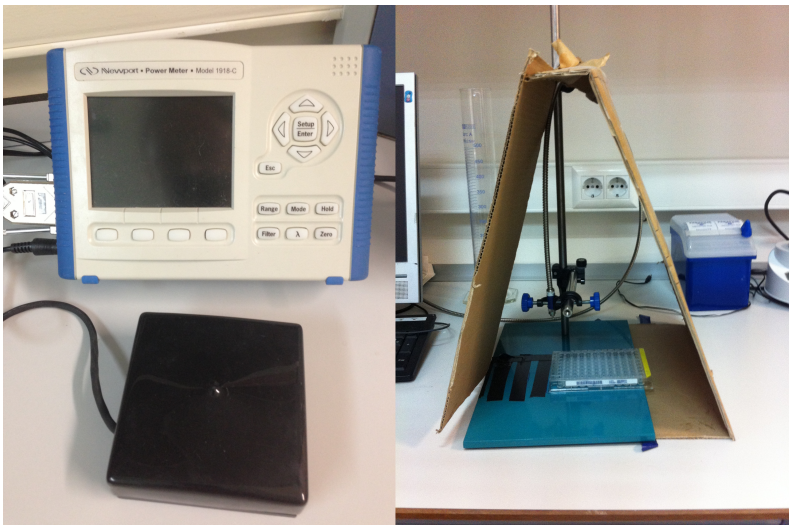

Figure 3.3 *Left:* Powermeter. *Right:* Application of laser irriadiation to the target cells.

- **Laser Groups**: In Laser groups L(xx) was used to signify the laser doses (xx) as(J/cm^2). *For example:* L(84) means that 84(J/cm^2)) laser dose was applied in the experiment.

  Laser Groups Design: L(84), L(168) and L(252)

  The following steps were applied in each experiments:

  1. P. aeruginosa ATCC 27853 were incubated at 37°C overnight, then incubated cells were centrifuged and resuspended in PBS, respectively.
  2. ICG solutions in specific concentrations were prepared by mixing with PBS.
  3. In the PDT and ICG groups, 50 μl aliquots of P. aeruginosa suspension were transferred into 96-well plate, then 50 μl ICG solution with a specific concentration was added into the wells and mixed with 50 μl of bacterial suspension in the wells of a 96-well plate.
  4. The laser setup was built to irradiate these specific wells. For this reason, bacterial suspension were transferred only into these cells.

5. After addition of ICG, mixture of bacteria and ICG into the wells were incubated at dark for 15 minutes.

6. In the Control and Laser groups, 50 μl bacterial suspension were transferred into 96-well-plate, then PBS with equal volume (50 μl) was added into these wells and mixed with bacterial suspension. Therefore the same conditions were applied to the all groups.

7. Then, the wells with bacteria and PBS in the Control and Laser groups were kept in the dark to be applied the same conditions with PDT and ICG groups.

8. After 15-minute incubation at dark, the laser irradiation was applied to the bacterial suspension in the PDT and Laser groups.

9. Viable cell counts were determined by serial dilution method after irradiation.

10. All diluted samples were plated on tyrptic soy agar.

11. These plates were kept at 37°C for 24 hours in dark room.

12. CFU (Colony-forming units) were counted to determine viable bacteria for each plate, after 24-hour incubation in dark.

13. All experiments were repeated at least three times, and all conditions were done in triplicates within each experiment.

3.5 Statistical Analysis

In each experiments, so as to keep the conditions constant, the same procedures were applied to control groups and experimental groups. Some of the wells are used for control groups and the others were used for experimental groups. Determined viable cell counts by serial dilution method were divided by the viable cell counts in control group. Therefore, on each 96-well plate, determined viable cell counts were normalized via getting ratio with corresponding control groups. In order to get statistical significance, normalized data were analyzed by one-way ANOVA and two-tailed- Student's

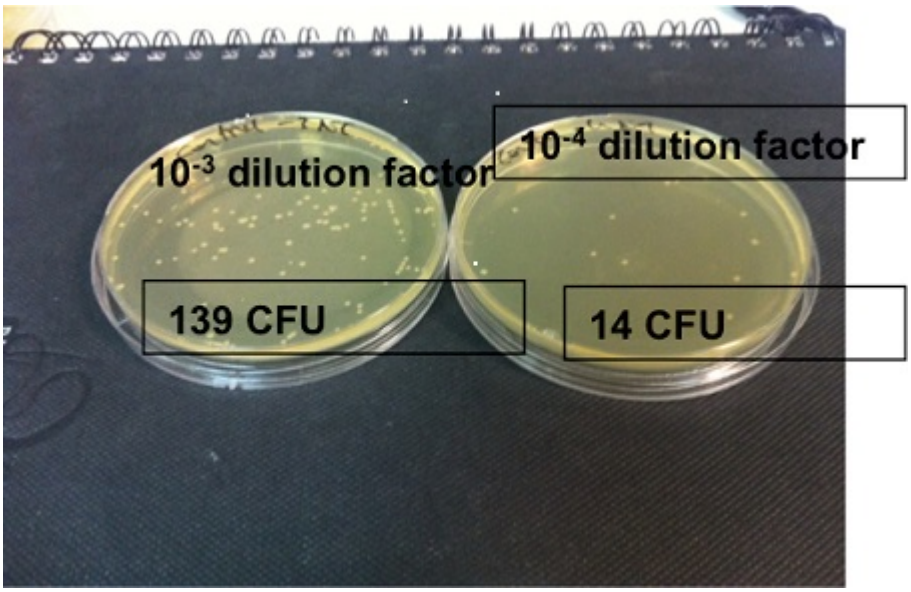

Figure 3.4 Diluted samples.

t-test. $p < 0.05$ values were accepted as statistically significant.

4. RESULTS

In this study, the main purpose was to demonstrate the importance of light and photosensitizer dose on cell viability of Pseudomonas aeuruginosa during photodynamic therapy process. The aim of this study was to investigate the biostimulative effect on bacterial cells when lower doses of laser and lower photosensitizer concentrations are applied.

In our prior experiments, the biostimulative effect on P. aeruginosa ATCC 27853 strain was observed instead of the PDT effect, when 84 J/cm^2 of energy dose (809-nm diode laser) was applied with 20, 50, 100, 125 and 150 μg/ml of ICG concentrations. When 84 J/cm^2 laser energy applied alone, around 10% increase in bacterial viability was established. The killing effect of PDT was obtained only on the higher concentrations such as 200 and 250 μg/ml (Figure 4.1). Killing effect was observed only on higher 200 and 250 μg/ml ICG concentrations as well (Figure 4.2).

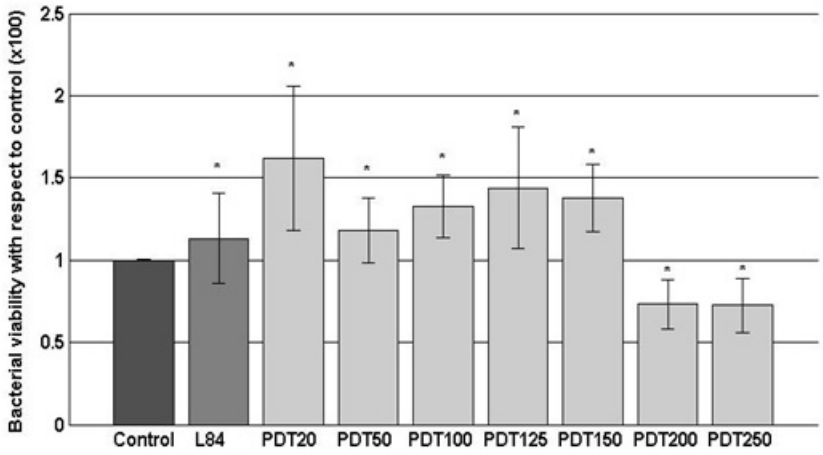

Figure 4.1 The percentage of viable P. aeruginosa ATCC 27853 cells in PDT groups. Viability of P. aeruginosa ATCC 27853 was determined after Laser only and PDT applications. Cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150, 200, 250 μg/ml). n>8 and * shows statistically significant groups with respect to control group ($p<0.05$)

In Figure 4.2, it was seen that there were statistically significant increase on P. aeruginosa ATCC 27853 cell proliferation in only two ICG groups (35% increase in 20 μg/ml ICG concentration and 25% increase in 250 μg/ml ICG concentration). With other ICG concentrations there were not any statistically significant changes. Contrary to expectations, there are a decrease in bacterial viability between 50-200 μg/ml of ICG group and the maximum decrease in bacterial viability in 100 μg/ml of ICG has been observed.

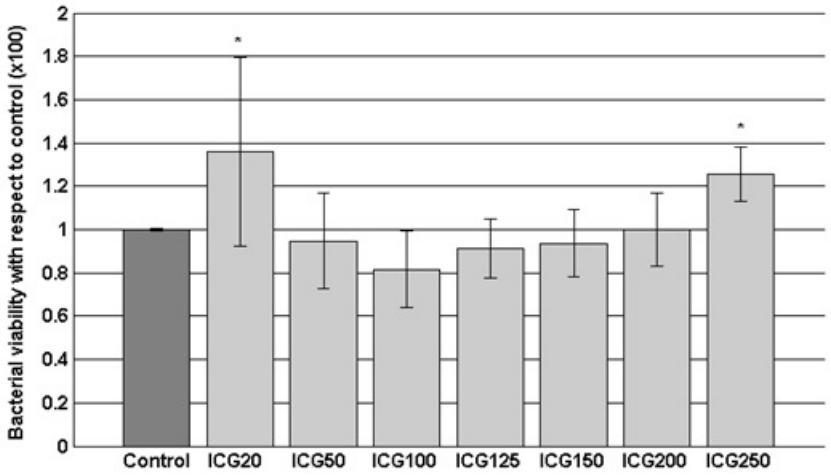

Figure 4.2 The percentage of viable P. aeruginosa ATCC 27853 cells in ICG Groups.ICG concentrations: 20, 50, 100, 125, 150,200, 250 μg/ml. Cell count in each experimental group was normalized with the untreated control group n>8 and * shows statistically significant groups with respect to control group ($p<0.05$)

After the results of prior experiments show that there may be a biostimulative effect of PDT at lower doses, we aimed to determine the safe PDT region via increasing laser energy doses with the same ICG concentrations (Light dose: 168J/cm^2 and 252 J/cm^2, ICG concentrations: 20, 50, 100, 125, 150, 200, 250 μg/ml).

In Figure 4.3, the increase of PDT groups are statistically significant amount cell proliferation (Light dose: 84J/cm^2 and 62% increase in 20 μg/ml ICG concentration, 18% increase in 50 μg/ml ICG concentration, 33% increase in 100 μg/ml ICG concentration, 43% increase in 125 μg/ml ICG concentration and 38% increase in 150

μg/ml ICG concentration, respectively). Although there was 27% decrease in bacterial viability in 200 and 250 μg/ml ICG concentration- PDT groups, the killing effect of PDT was not sufficient.

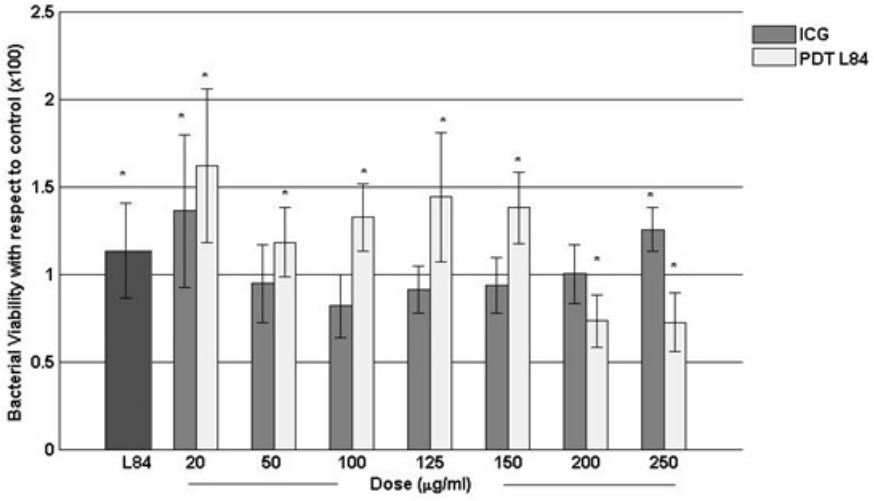

Figure 4.3 The percentage of viable P. aeruginosa ATCC 27853 cells in PDT Groups. Cell count in each experimental group was normalized with the untreated control group (Light dose: 84 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150, 200, 250 μg/ml). n>8 and * shows statistically significant groups with respect to control group ($p<0.05$)

In Figure 4.4, it was seen that there were statistically significant increase on P. aeruginosa ATCC 27853 cell proliferation in only 20 μg/ml ICG concentration with 168 J/cm^2 energy dose as 27% increase. When 168 J/cm^2 energy dose was applied alone, the light caused a 15% decrease in viable cell count. The biostimulative effect of PDT was seen at lower ICG concentrations with the same level energy dose application. In 50 μg/ml ICG concentration with 168 J/cm^2 energy dose, the cell number is almost the same with untreated control group. The killing effect of PDT was seen over 100 μg/ml ICG concentrations with 168 J/cm^2 energy dose. And the killing effect of PDT on these higher concentrations were 99% efficacy.

In Figure 4.5, 252 J/cm^2 of energy dose was applied with the same ICG concentrations 20, 50, 100, 125, 150, 200, 250 μg/ml). When light as 252 J/cm^2 energy dose was applied alone, there was not any significant change in cell amount. But the energy

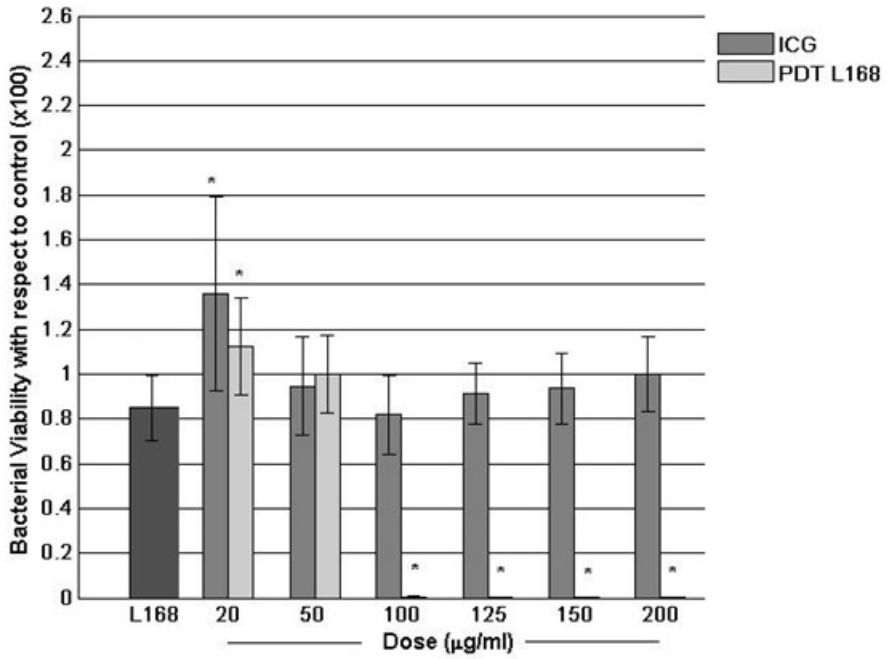

Figure 4.4 The percentage of viable P. aeruginosa ATCC 27853 cells in PDT Groups. Cell count in each experimental group was normalized with the untreated control group (Light dose: 168 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150, 200 μg/ml). n>8 and * shows statistically significant groups with respect to control group ($p<0.05$).

dose was applied with 20 μg/ml ICG concentration, the significant increase on bacterial cell count was observed. The increase was 41%. When the energy dose was applied with 50 μg/ml ICG concentration, there was no significant change on viable cell count, it was almost same as untreated control group. Over 100 μg/ml ICG concentrations, the desired bactericidal effect of PDT was seen. The killing effect of PDT in 150 μg/ml ICG concentrations was observed as 99% efficacy.

The amount of dose of light and photosentsitizer concentration must be adjusted efficiently to prevent proliferation of bacterial cells during PDT applications. But, when light energy dose and concentration of photosensitizer are low, it could cause oxidative stress on cells and this triggers some pathways that result in biostimulative effect because of low concentration of free oxygen radicals produced after PDT application. When PDT is applied with lower doses, the produced oxygen radicals will be low and it causes insufficient bactericidal effect of PDT and it may lead to biostimulative effect. Experiments show that the PDT dosimetry should be applied at optimum dose

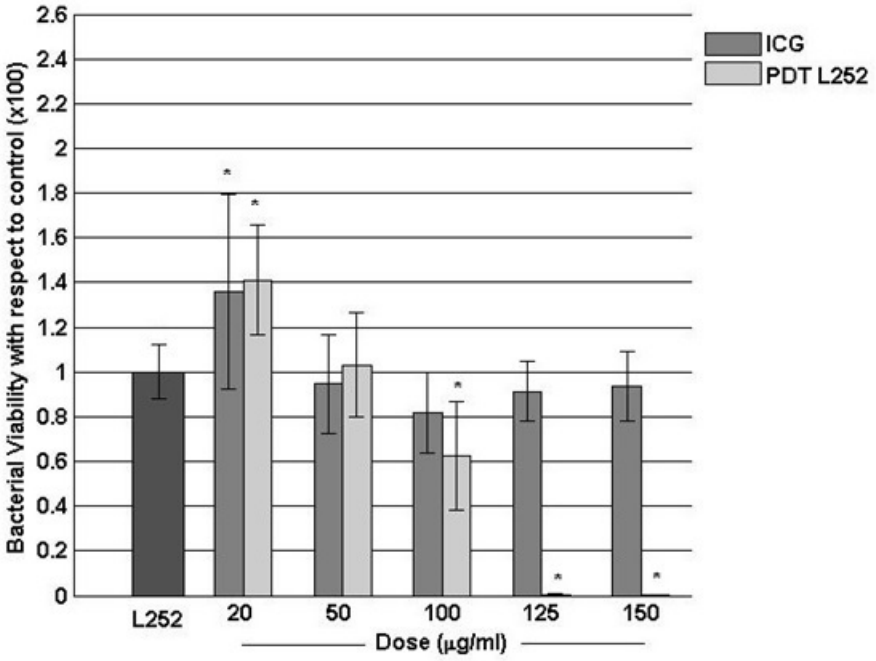

Figure 4.5 The percentage of viable P. aeruginosa ATCC 27853 cells in PDT Groups. Cell count in each experimental group was normalized with the untreated control group (Light dose: 252 J/cm^2 and ICG concentrations: 20, 50, 100, 125, 150 μg/ml). n>8 and * shows statistically significant groups with respect to control group ($p<0.05$).

in both light and concentration doses, otherwise the unexpected biostimualtive effect on bacteria cell could be resulted in instead of bactericidial effect of PDT.

5. DISCUSSION

The PDT mechanism includes a combination of appropriate light energy, a photosensitizer which has a molecule that absorbs the light energy and the presence of molecular oxygen which causes the killing effect to the target cell. The main success of PDT for selectivity and efficacy is dependent on PDT dosimetry which is described within four parameters: Light dosage, photosensitizer concentration, and the amount of produced reactive oxygen species and the PDT time interval (duration between light irradiation and photosensitizer addition) [4], [44], [61],[62], [65], [102]. These reactive oxygen species have an important role on cell death. Applied photosensitizer concentration and laser energy dose are critical in PDT applications. During the photoactivation process, reactive oxygen species are produced when a photosensitizer is activated by the appropriate light energy. For this reason, the time interval is important as photosensitizer concentration and light energy dose as about ROS production. The produced ROS reacts with biological cells and destroys the target cell [56], [102], [106]. But some studies show that low level light or low concentration of oxygen radicals may lead to cell proliferation with various biochemical pathways instead of bactericidal effect of PDT. When PDT is applied with lower doses, produced oxygen radicals will be low and it leads to insufficient killing effects on bacteria such as still-living tumor responses or cell proliferation [64], [68], [21]. So, if the dose of light and the concentrations of photosensitizers are low, it could cause oxidative stress on cells and this triggers some pathways that could end up with biostimulative effects due to low concentration of free oxygen radicals produced after PDT application.

The main purpose of this study was to investigate the PDT safe region for bactericidal application and to demonstrate the importance of PDT dosimetry. PDT dose must be optimized properly to overcome the multidrug resistant bacteria problem on wounds, otherwise biostimulation effect could occur on bacteria instead of the killing effect PDT. In our study, indocyanine green (ICG) as a photosensitizer and 809-nm

diode laser as a light source were used in different doses to determine the bactericidal region of PDT on P. Aeruginosa ATCC27853 strain. Laser energy dose (84 J/cm^2, 168 J/cm^2 and 252 J/cm^2) were applied with different ICG concentrations (20, 50, 100, 125, 150, 200 and 250 μg/ml). The data showed that lower ICG concenrations with lower light doses caused cell proliferation on P. Aeruginosa ATCC27853 instead of cell death. When we increased the ICG concentration, killing effect was observed but it was not efficient enough to kill 99% bacteria. When we increased the light dose, the expected killing effect of PDT was occured lower doses. It determined the safe region and it showed that the PDT dosimetry should be optimized in particular for each bacterial strain.

In our study, we highlighted that there could be biostimuation on bacteria cell if PDT dosimetry is applied at lower levels. When 84 J/cm^2 energy dose was applied with lower ICG concentrations (20, 50, 100, 125 and 150 μg/ml), cell proliferation was observed on P.Aeruginosa contrary to expected killing effect of PDT. When energy dose increased to 168 J/cm^2, cell proliferation was observed with lower ICG concentrations (20-50 μg/ml). From 100 μg/ml ICG concentration with 168 J/cm^2 combination, bactericidal effect was observed.

When we applied a lower laser energy dose alone (84 J/cm^2), the cell proliferation was obtained at 10%. Low level laser therapy can cause cell proliferation too [66], [67]. When we applied ICG alone, the lowest (20 μg/ml) and the highest ICG concentrations (250 μg/ml) increased cell viability like the similar study of Sato [108]. Under oxidative stress, some cell pathways might have been activated with low ICG concentration as cellular response. But the biostimulative effect on 250 μg/ml ICG concentarion was unexpected situation. These kind of cellular responses related to ICG are needed to investigate.

The main aim of antibacterial PDT is to eradicate the multidrug resistant bacteria. The results showed that PDT dose has very critical effect on infecting bacteria. When PDT dosimetry are not optimized properly, it can cause biostimulative effect. The amount of dose of light and photosentsitizer concentration must be adjusted efficiently to prevent proliferation of bacterial cells.

6. CONCLUSION

Antibacterial photodynamic therapy is an alternative method to combat with antibiotic resistant bacteria. But our results show that antibacterial photodynamic therapy can either have the biostimulative effect at lower doses of photosensitizer and light or have desired killing effect of PDT at higher doses. PDT dosimetry should be optimized properly for each pathogen and should be determined the safe region before theraupatic use. For this reason, the parameters of PDT have to be chosen well for bactericidal effect and prevention any cell proliferation of bacteria.

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