G. KADIKÖYLÜ

IZMIR KATIP CELEBI UNIVERSITY

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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE EFFECT OF ANTIBACTERIAL PHOTODYNAMIC THERAPY ON HEALTHY FIBROBLAST AND KERATINOCYTE CELLS

M.Sc. THESIS

Gülce KADIKÖYLÜ

Department of Biomedical Technologies

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Thesis Advisor: Assist. Prof. Dr. Nermin TOPALOĞLU AVŞAR

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İZMİR KATİP ÇELEBİ ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ANTİBAKTERİYEL FOTODİNAMİK TERAPİNİN SAĞLIKLI FİBROBLAST VE KERATİNOSİT HÜCRELERİ ÜZERİNDEKİ ETKİSİ

YÜKSEK LİSANS TEZİ

Gülce KADIKÖYLÜ
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OCAK 2019

Gülce KADIKÖYLÜ, a **M.Sc.** student of **IKCU Graduate School Of Natural And Applied Sciences**, successfully defended the thesis entitled "**The Effect Of Antibacterial Photodynamic Therapy On Healthy Fibroblast And Keratinocyte Cells**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Assist. Prof. Dr. Nermin TOPALOĞLU AVŞAR**
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Date of Submission : 03.01.2019
Date of Defense : 16.01.2019

To my family,

FOREWORD

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ABBREVIATIONS

µg	**:** microgram
AO/PI	**:** Acridine Orange/Propidium Iodide
aPDT	**:** Antibacterial Photodynamic Therapy
C	**:** Control group
Ce6	**:** Chlorin e6
cm^2	**:** square centimeter
CO_2	**:** Carbon dioxide
DMEM	**:** Dulbecco's Modified Eagle's Medium
DMSO	**:** Dimethyl Sulfoxide
EDTA	**:** Ethylenediaminetetraacetic acid
FBS	**:** Fetal Bovine Serum
H_2O_2	**:** Hydrogen peroxide
HS2	**:** Healthy human skin keratinocyte cell line
ICG	**:** Indocyanine Green
J	**:** Joule
L	**:** Laser group
L929	**:** Healthy mouse skin fibroblast cell line
LED	**:** Light Emitting Diode
MB	**:** Methylene blue
MDA	**:** Malondialdehyde
ml	**:** milliliter
MRSA	**:** Methicillin-resistant *Staphylococcus aureus*
MTT	**:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NIR	**:** Near-infrared
nm	**:** nanometer
^oC	**:** Centigrade
PBS	**:** Phosphate Buffered Saline
PDT	**:** Photodynamic Therapy
PS	**:** Photosensitizer
ROS	**:** Reactive Oxygen Species
SCC	**:** Squamous Cell Carcinoma
TBO	**:** Toluidine blue
W	**:** Watt

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THE EFFECT OF ANTIBACTERIAL PHOTODYNAMIC THERAPY ON HEALTHY FIBROBLAST AND KERATINOCYTE CELLS

ABSTRACT

Antibacterial photodynamic therapy is a treatment that occurs in the presence of light, photosensitizer and oxygen. The photosensitizer reacts with the molecular oxygen in the environment when it is stimulated by light at the appropriate wavelength, causing reactive oxygen species at the end. Reactive oxygen species show high cytotoxic effects on cells and cause necrosis or apoptosis. In recent years, the resistance of bacteria to antibiotics has caused infection treatments to become more difficult. This situation has become a threat to human and animal health. Therefore, antibacterial photodynamic therapy has been an alternative approach for the photoinactivation of pathogens in the treatment of infection. The only important issue in this method is not how high rate of pathogens are killed, but how healthy cells that play an important role in wound healing are affected by antibacterial photodynamic therapy. From this perspective, in this study the effects of indocyanine green and 808-nm wavelength diode laser whose lethal effect on some pathogens has previously been proven, on healthy skin fibroblast and healthy skin keratinocyte cells have been investigated. Photodynamic therapy which is intended to be used in clinics, the main objective of this study is to determine the dose of light and drug concentrations that will not harm healthy cells but will have cytotoxic effects on pathogens.

In this study, the effect of photodynamic therapy on healthy fibroblast and keratinocyte cells was investigated *in vitro*. The effects on the cells after the applications, using the drug and light doses which was proved to be able to kill pathogens, were examined by MTT Analysis, Lipid Peroxidation Analysis and Acridine Orange / Propidium Iodide Staining. While healthy skin fibroblast cells and healthy skin keratinocyte cells were not only adversely affected by laser application, indocyanine green damaged the cells when applied at high concentrations. Photodynamic therapy applications using indocyanine green at 4-125 µg/ml concentrations and 84 J/cm^2 energy dose did not damage keratinocyte cells significantly. Application of 252 J/cm^2 energy dose damaged keratinocyte cells with all indocyanine green concentrations. In photodynamic therapy applications on fibroblast cells, two different energy doses and 4 µg/ml and 10 µg/ml indocyanine green concentrations did not damage the cells significantly.

As a result of these applications, it has been understood that antibacterial photodynamic therapy applications should be applied by optimizing the light and drug doses. Healthy cells can be highly damaged if only photoinactivation of the pathogens is aimed during photodynamic therapy. When the dose optimization is achieved properly in this method, successful photoinactivation of the pathogens will be ensured and the damage of healthy cells in the surrounding tissue can be prevented or minimized.

ANTİBAKTERİYEL FOTODİNAMİK TERAPİNİN SAĞLIKLI FİBROBLAST VE KERATİNOSİT HÜCRELERİ ÜZERİNDEKİ ETKİSİ

ÖZET

Antibakteriyel fotodinamik terapi ışık, ışığa duyarlı ilaç (fotosensitizer) ve oksijenin varlığında gerçekleşen bir tedavidir. Fotosensitizan uygun dalga boyundaki ışık ile uyarıldığında ortamdaki moleküler oksijen ile reaksiyona girerek reaktif oksijen türlerinin ortaya çıkmasına neden olur. Reaktif oksijen türleri hücrelerde oldukça sitotoksik etkiler gösterir ve hücrelerin ölmesine neden olur. Son yıllarda bakterilerin antibiyotiklere karşı direnç geliştirmesi enfeksiyon tedavilerinin zorlaşmasına neden olmuştur. Bu durum insan ve hayvan sağlığını tehdit eden bir sorun haline gelmiştir. Bu sebeple antibakteriyel fotodinamik terapi, enfeksiyon tedavisinde patojenlerin fotoinaktivasyonunu sağlayan alternatif bir yaklaşım olmuştur. Bu yöntemde tek önemli konu patojenlerin yüksek oranda öldürülmesi değil, yara iyileşmesinde önemli rolü bulunan sağlıklı hücrelerin antibakteriyel fotodinamik terapiden nasıl etkilendiğinin de bilinmesidir. Bu bakımdan, bu çalışmada bazı patojenler üzerindeki öldürücü etkisi daha önce kanıtlanmış olan indosiyanin yeşil maddesinin ve 808-nm dalga boyunda ışıma yapan diyot lazerin sağlıklı deri fibroblast ve sağlıklı deri keratinosit hücreleri üzerindeki etkisi araştırılmıştır. Kliniğe taşınmak istenen fotodinamik terapi yönteminde sağlıklı hücrelere zarar vermeyecek ama patojenler üzerinde sitotoksik etkiler gösterecek ışık dozu ve ilaç konsantrasyonlarının belirlenmesi bu çalışmanın temel amacıdır.

Bu çalışmada fotodinamik terapinin etkisi sağlıklı fibroblast ve keratinosit hücreleri üzerinde *in vitro* ortamda incelenmiştir. Patojenleri öldürebildiği kanıtlanan ilaç ve ışık dozları kullanılarak gerçekleştirilen uygulamalardan sonra hücrelere olan etkiler MTT Analizi, Lipid Peroksidasyon Analizi ve Akridin Turuncusu/Propidyum İyodür Boyaması yapılarak incelenmiştir. Sağlıklı deri fibroblast hücreleri ve sağlıklı deri keratinosit hücreleri sadece lazer uygulamasından olumsuz yönde etkilenmezken, indosiyanin yeşil yüksek konsantrasyonlarda uygulandığında hücrelere zarar vermiştir. 84 J/cm^2 enerji dozu ile 4- 125 µg/ml aralığındaki konsantrasyonlarda indosiyanin yeşil kullanılarak yapılan fotodinamik terapi uygulamaları keratinosit hücrelerine zarar vermemiştir. 252 J/cm^2 enerji dozu uygulanması keratinosit hücrelerine tüm indosiyanin yeşil konsantrasyonlarında zarar vermiştir. Fibroblast hücreleri üzerindeki fotodinamik terapi uygulamalarında ise 4 µg/ml ve 10 µg/ml indosiyanin yeşil konsantrasyonu ile iki farklı enerji dozu hücrelere zarar vermemiştir.

Bu uygulamalar sonucunda, fotodinamik terapi uygulamalarının ışık ve ilaç dozlarının optimizasyonu sağlanarak gerçekleştirilmesi gerektiği anlaşılmıştır. Fotodinamik terapi sırasında sadece patojenlerin öldürülmesi hedeflendiğinde sağlıklı hücreler zarar görebilmektedir. Kliniğe taşınmak istenen bu yöntemde doz optimizasyonu sağlandığında patojenlerin başarılı şekilde fotoinaktivasyonu sağlanırken çevre dokuda bulunan sağlıklı hücrelerin zarar görmesi engellenebilecektir.

1. INTRODUCTION

Rapid increase in antibiotic resistance and the inadequacy of other antibacterial treatments are serious risk factors for human and animal health worldwide. Researchers have begun to focus on photodynamic therapy as an alternative treatment. This treatment is based on the killing of pathogens by the formation of toxic products after the interaction of light with light-sensitive drugs (photosensitizer) and oxygen.

It is also important to emphasize how antibacterial photodynamic therapy affects healthy cells which play an important role in wound healing process. In this regard, the previously proven antibacterial effect of indocyanine green and 808-nm on healthy mouse fibroblast cells and healthy human keratinocyte cells was investigated in this study.

The main objective of this research is to determine the concentrations of indocyanine green and the energy dose of laser light which are destructive to certain pathogens and not harmful to healthy fibroblasts and keratinocytes.

1.1 Photodynamic Therapy

The discovery of photodynamic effect depends on the early 1900s. After its discovery, preliminary studies were carried out in Europe. Despite these studies, both clinical and application studies have not reached to a sufficient level until the 1980s. In the 1990s, studies of the treatment of malignant and pre-malignant diseases by photodynamic therapy were started seriously [1].

Photodynamic therapy is a form of treatment that produces reactive oxygen species using a non-toxic substance called a photosensitizer and appropriate wavelength that allow this substance to react with molecular oxygen, and leads to the death of the target cell [2].

Photodynamic therapy is a promising treatment that can be used for cancer treatment or antibacterial therapy. The photosensitizer drugs used in these treatments are

administered intravenously or topically and accumulate in the target area. When the light at the appropriate wavelength to activate the photosensitizer is applied to the target site, the presence of oxygen in the medium induces necrosis or apoptosis in the cells by forming free radicals or reactive oxygen species, thereby causing cell or tissue damage [3] (Figure 1.2).

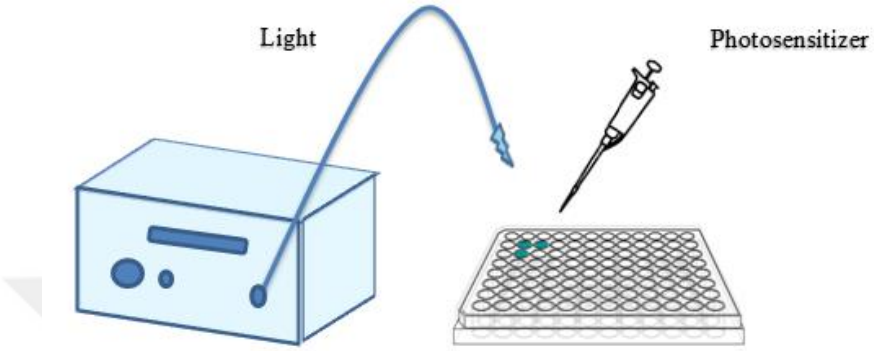

Figure 1.1 : Schematic representation of light and photosensitizer application to cells.

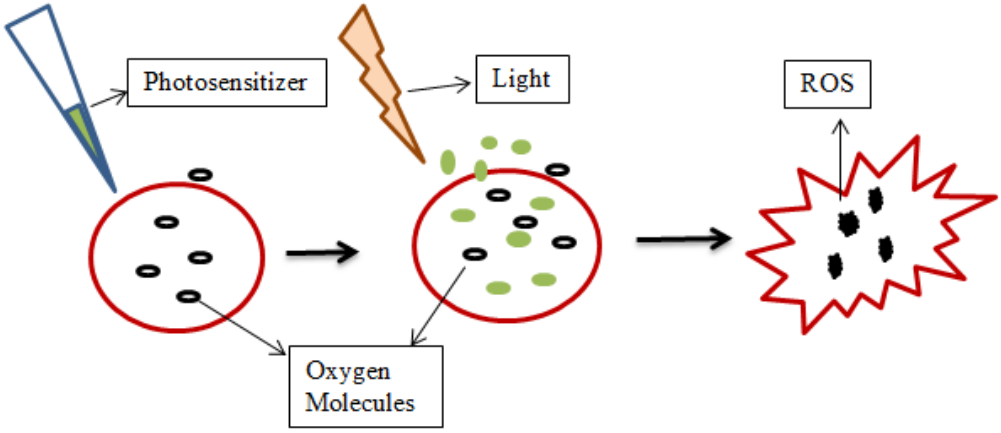

Figure 1.2 : Schematic representation of Photodynamic Therapy.

Conventional treatment methods might be insufficient in many fields such as cancer treatment or antibacterial therapy. Therefore scientists have turned to alternative treatment methods in the last century. Photodynamic therapy is an important alternative which has been investigated because of its advantages of showing minimal side effects to surrounding tissues and using it together with traditional treatment methods [4].

1.2 Mechanism of Photodynamic Therapy

There should be three basic elements in the mechanism of photodynamic therapy. These are photosensitive substance called photosensitizer (PS), light which has appropriate wavelength, and molecular oxygen [5]. The absorption of light by the light sensitive drug provides the formation of cytotoxic products which perform the desired therapeutic effect. Oxygen radicals are cytotoxic products that kill the target tissue [6].

The initial phase of the photodynamic therapy mechanism is the absorption of the light that is at appropriate wavelength by the photosensitizer. The electrons of the drug molecule located at the ground state absorb the light and rise to the excited singlet level. The electrons present here have a very short half-life of 10^{-6} to 10^{-9} sec. The electrons in this stage either fall to the ground state with fluorescence or cross to the triplet state with inter system crossing. The half-life of electrons at triplet state is 10^{-3} sec. and this value is about 3 times higher than the excited singlet state. This time is enough for the electrons to interact with the molecular oxygen in the environment. The photo-oxidative reaction formed at this stage is observed in two types. Type 1 mechanism is based on the transfer of electrons in the photosensitizer molecule. This results in reactive oxygen species such as hydrogen peroxide and hydroxyl radicals. In the type 2 mechanism, the energy of the photosensitizer in the triplet state is transferred to the directly molecular oxygen. This situation ends with the formation of singlet oxygen [7; 8; 9] (Figure 1.3). Singlet oxygen causes damage to the cell structures such as DNA, lipids and proteins [8].

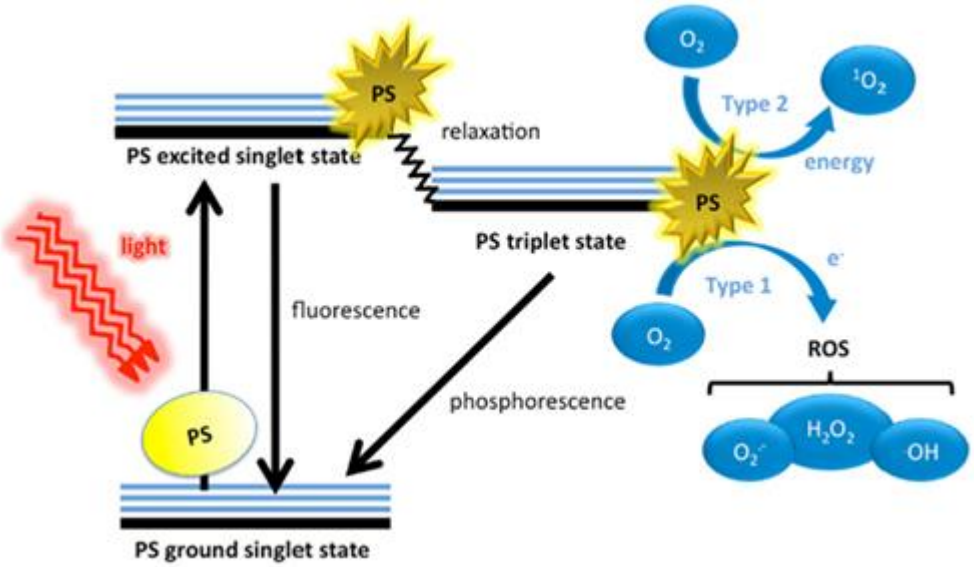

Figure 1.3 : Schematic illustration of mechanism of Photodynamic Therapy [10].

1.3 Purpose of Photodynamic Therapy

Photodynamic therapy is an alternative treatment method that can be widely used in cancer treatment and antibacterial therapy. New photosensitizers and clinical developments of PDT have increased the interest in this treatment [11; 12; 13].

1.3.1 Cancer treatment

PDT, which can be used in the treatment of tumor, can still be considered a new and promising method. PDT, which is successful in the treatment of neoplastic and non-malignant diseases, provides a significant improvement in the standard of the live of patients [14].

There must be 3 different non-toxic factors in PDT: they are photosensitizer, light source that emits visible or near-infrared wavelength and molecular oxygen dissolved in target tissue [11]. These elements, which do not show toxic effects alone, result in highly reactive singlet oxygen production and this toxicity leads to cell death by apoptosis or necrosis [14]. In cancer treatment, the photosensitizer applied intravenously or topically accumulates in the target tissue and the light with appropriate wavelength to the photosensitizer is held in the target region. Thus, the localized destruction of the cancerous tissue is ensured.

Photosensitizer shows a special affinity against the tumor. This specific binding model between tumor cells and photosensitizers can be explained by the low pH of the tumor tissue, receptor expression caused by high LDL, insufficient developed

lymphatic drainage, and the tendency of the tumor tissue to create new vessels [11; 15; 16]. It is known that the tumor is destroyed by three related pathways with PDT (Figure 1.4). These; causing direct tumor cell death, damaging the vascularity of the tumor tissue, and inducing inflammatory reactions to induce the development of the immune response of the system [14; 15; 17].

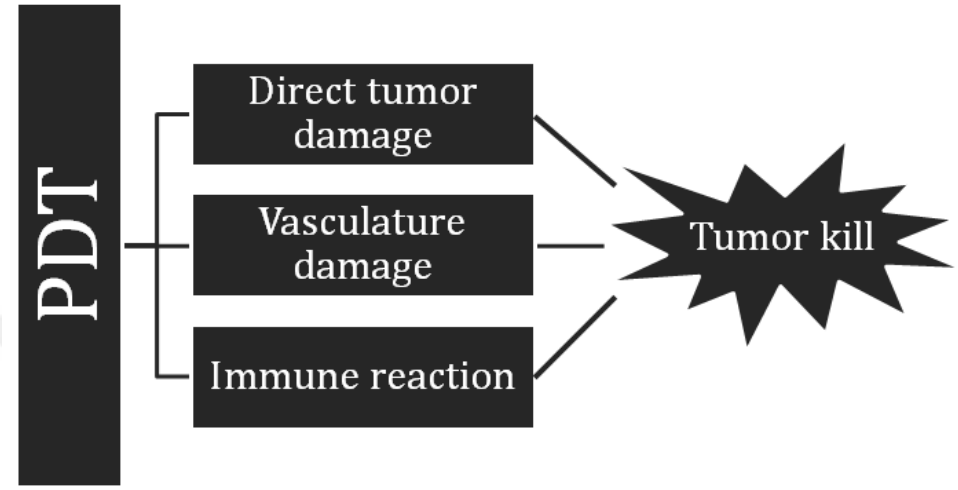

Figure 1.4 : The pathways of killing the tumor with Photodynamic Therapy.

PDT can be used as a permanent treatment in early-stage cancers while providing palliative treatment in advanced cancers. It can also be used as an adjuvant treatment in addition to conventional therapies [17]. Advantages of PDT compared to surgery, chemotherapy or radiotherapy; it reduces long-term morbidity and does not impede the selection of future treatment modalities for residual or possible recurrent disease. Furthermore, while the efficacy of chemotherapy or radiotherapy method in the event of recurrence of disease is impaired during their use, PDT can be repeated for patients without deteriorating its efficacy in the future. These are important limiting factors for chemotherapeutics and radiotherapy [14].

1.3.2 Antibacterial therapy

Antibacterial PDT enables inactivation of microorganisms without creating microbial resistance [13]. The development of resistance of bacteria against antibiotics used in antibacterial treatment and the presence of a wide variety of pathogens lead to difficulties in this treatment. In this respect, it has become a necessity to find new treatment methods and new drugs. Systemic use of antibiotics causes damage to the beneficial flora found in our body. In this respect, since PDT is used for localized infections, it does not harm the beneficial flora.

At the same time, the application of PDT with systemic antibiotic therapy can alleviate the cost of treatment and accelerate the healing process [18].

The photosensitizer used in antibacterial PDT passes through the cell wall of the microorganism and penetrates into the cell. When the light with a specific wavelength which activate this photosensitizer illuminates the target cell, it interacts with the oxygen in the environment and leads to the formation of reactive oxygen species containing free radicals and singlet oxygen and this treatment damages the target cell [12; 13; 19]. Reactive oxygen species formed during photodynamic therapy are thought to interact directly with bacterial cells or to enter cell and interact with cell membrane internal structures [20; 21; 22].

Wavelengths in visible and infrared spectrum are mostly preferred for photodynamic therapy. Generally light sources that radiate in the visible spectrum are preferred in antibacterial studies [23]. Visible light has limited penetration capacity for biological tissue. The wavelengths in the infrared spectrum is more advantageous in this respect. For example, while 650-nm can progress 3-3.5 mm in the tissue, 809-nm can progress 6 mm in the tissue [24]. For this reason, it is thought that the wavelengths in the infrared spectrum and the photosensitizers that absorb them well are considered to be more successful in the treatment of infections that have progressed deeper into the tissue.

Antibacterial PDT is a painless and non-invasive, broad-spectrum, low-cost, promising treatment that can be used in different types of infections compared to antibiotic therapy [19]. The success of PDT depends on the various parameters such as the concentration of the photosensitizer used, the incubation time of the photosensitizer applied, the wavelength and power of the light source, and the type of the photosensitizer [12; 25].

1.4 Different Applications of Photodynamic Therapy

While photodynamic therapy (PDT) has continued its development on benign and malignant tumor treatment, it has been found that PDT has more successful effect in diseases other than cancer. Today, PDT is an alternative treatment against antibiotic resistance in infection treatments. The antimicrobial applications of PDT have given positive results. Antibacterial, antifungal effect is also outstanding in the treatment of infection [26].

1.4.1 Antimicrobial applications of PDT

Antimicrobial PDT is a treatment to destroy pathogens that can cause localized infections such as dental diseases, dermal infections caused by acne, wounds and burns [20].

Wong et al. performed a study to investigate the antibacterial effect of photodynamic therapy. In this study, they used Indocyanine Green (ICG) as a photosensitizer and Near-infrared (NIR) led lamp as light source. They tried different concentrations of ICG and different light doses on 4 different types of Methicillin-resistant *Staphylococcus aureus* (MRSA). According to the results, they determined that 200 J/cm^2 light dose and 100 µg/ml ICG concentration provides 99.999% inhibition in bacterial growth. When the ICG concentration and the light dose were reduced to 25 µg/ml and 100 J/cm^2 respectively, and 0.01% H_2O_2 was added, they reported that the same rate of inhibition was achieved and these values did not harm the fibroblast cells [27].

Azizi et al. investigated the inhibitory effect of PDT on *Lactobacillus acidophilus* bacteria using light which has wavelength of 660 nm and 808 nm. As a photosensitizer, they used Methylene blue (MB) and ICG which can be stimulated at those wavelengths, respectively. In their study, they also investigated the effects of penicillin, chlorhexidine and sodium hypochlorite. According to the results, the PDT group with 660 nm of wavelength (energy density 1.88 J/cm^2) and MB showed more inhibition effect on the bacteria than the PDT group with 808 nm of wavelength (energy density 9.48 J/cm^2) and ICG. Other chemical agents applied in other groups provided complete bacterial inhibition. As a general result of the study, the MB and the PDT groups with the laser application of 660 nm provided an effective inactivation for this bacterial strain [28].

Mima et al. investigated the effect of PDT on some Candida species which are frequently encountered in oral infections. In this study, standardized dentures were infected with Candida species. Blue light emitting LED and 50 mg/L concentration of Photogem as a photosensitizer were used to provide photodynamic inactivation. As a result, they showed a significant decrease in all Candida species compared to the control group [29].

Azizi et al. examined the effect of nystatin as traditional treatment agent, MB and ICG as photosensitizers on *Candida albicans* species to show the antifungal effect of PDT. The most effective PDT group that eliminated the C. albicans species was determined as the group which uses ICG and the 808 nm of wavelength [30].

1.4.2 Anticancer applications of PDT

Photodynamic therapy is a method that can be applied alone or as a palliative treatment by traditional treatment methods [1]. Especially in the treatment of early cancers, PDT can be applied for the treatment of macular degeneration, skin [31], larynx, mouth, head and neck, lung, brain cancers [1].

Mamoon et al. investigated the effect of PDT for the treatment of skin melanoma cells. They reported PDT as an effective method for superficial cancers. For this study, they used ICG stimulated by NIR laser light source which has 788 nm of wavelength. They incubated human skin melanoma cells with ICG at a concentration of 150 µg/ml and applied laser multiple times. They reported that apoptosis was triggered after 15 minutes of laser application. In this study, it was concluded that ICG-PDT was effective in topical treatment of melanoma cells [32].

Aydoğan et al. performed PDT application on MRC-5 cells which are healthy lung cells and MIAPaCa-2 cells which are pancreatic cancer cells. They evaluated the difference between PDT application on healthy cells and cancer cells. They used a diode laser which has a wavelength of 670 nm with an energy dose of 5 J/cm^2 and Ce6 at a concentration of 10 µM. It was determined that cell viability was decreased significantly in Ce6 applied PDT group compared to the other groups. After treatment, 67% of normal cells remained alive [33].

Shi et al. conducted a study on Eca-109 cells belonging to human esophageal cancer with PDT application. Cells were incubated with different concentrations of Sinoporphyrin sodium (DVDMs) and illuminated with a laser which has 650 nm of wavelength with different energy doses. They observed significant differences in cell viability between the experimental and the control groups. They reported that PDT treatment with DVDMs stimulated apoptosis and autophagy in esophageal cancer cells [34].

Toratani et al. applied PDT treatment using photofrin as a photosensitizer and a laser light source of 630 nm of wavelength to the patients of oral cancer. In 34 patients, superficial oral squamous cell carcinoma (SCC) was treated with PDT and the effects of the applications were evaluated. In the evaluation performed 6 months after the single application, 30 of 34 patients showed complete response, 3 patients showed partial response, 1 patient had no change. In this treatment method, the researchers reported that oral structure and functions were preserved, there were very few side effects in the patients and good cosmetic results [35].

1.5 Advantages of PDT Applications

In recent years, PDT has been used especially for the treatment of cancer and many infections.

According to researches, PDT is more effective in early cancers [36]. Intravenously or topically applied photosensitizer accumulates selectively in tumor tissue. The applied photosensitizing drug does not have toxic effect alone but it causes damages only to cancerous tissue when light is applied on it after accumulation of the drug in these cells. This reduces the possibility of the damage to the surrounding healthy tissues [7]. Since there is no surgical condition, the postoperative complications are eliminated. In addition, it is a non-invasive or minimally invasive treatment and does not cause scar tissue. As there is no surgical intervention, there is no tissue loss and it provides a cosmetic advantage for the patients. The side effects of other treatment modalities used in cancer treatment in the whole body can be eliminated in PDT. Chemotherapy affects the entire system, PDT acts locally. It is also possible to combine it with other conventional treatment methods such as chemotherapy [37]. PDT is a repeatable technique and the risk of developing resistance is low. This prevents the formation of metastasis of the tumor tissue and does not cause immune system suppression [14].

For the antibacterial purposes, PDT provides a number of advantages compared to antibiotics, which are generally topically applied and have a local effect in the treatment of infection. PDT, which can be used in the treatment of many infections, can be repeated and does not harm the patients. One of the most important advantages is that pathogens do not develop resistance. Nowadays, antibiotic resistance which becomes the most important problem for the treatment of infection can be overcome with this treatment method [38; 39]. It is also a broad-spectrum treatment, effective not only on bacteria but also on fungi, parasites and viruses [20; 40**]** (Figure 1.5).

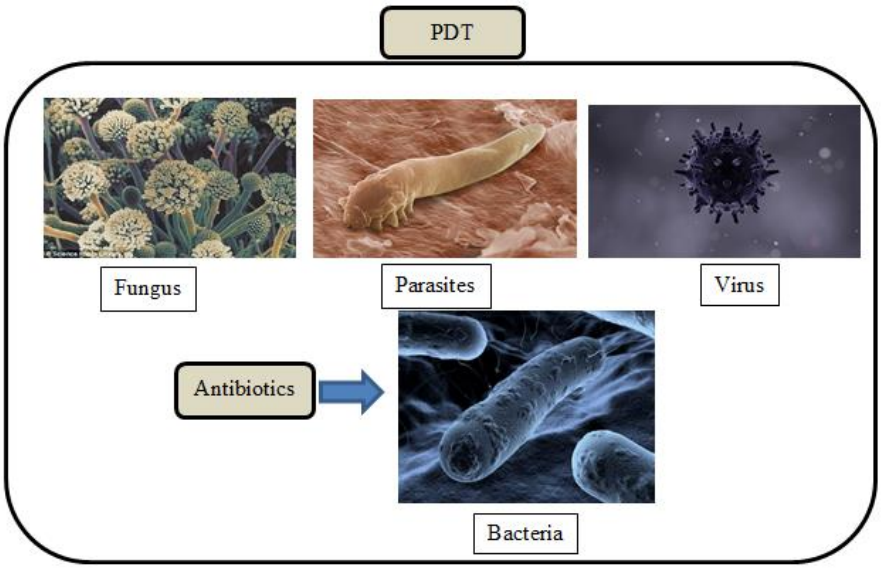

Figure 1.5 : The figure represents a broad spectrum of photodynamic therapy. While antibiotics are effective only on bacteria, PDT also acts on other pathogens.

Another advantage for both cancer and infection treatment is that it is a low cost treatment. It is also a quicker and an easier method than others [38; 40].

1.6 Disadvantages of PDT Applications

Although the photosensitizer used in PDT has a high selectivity to the cancerous or infectious tissue, it also takes a certain period of time to be removed from other healthy tissues. During this time, patients' exposure to light can cause irritation on their body [37]. Rarely can be seen blister formation and stain on the skin [41]. In order to eliminate this side effect, further studies should be conducted towards tissue-specific targeting of the photosensitizer [37].

PDT is a localized treatment method. Particularly, the most important factor in the death of cancer patients is cancer spread to other parts of the body by metastasis. Therefore, PDT is insufficient in systemic therapies [42].

Many types of photosensitizers are available. The wavelength and the degree of effectiveness of each photosensitizer are different. For this, it is important to determine the appropriate photosensitizer in each treatment and to adjust the appropriate dosimetry for the patient. The discovery of new drugs and the opening of treatment centers for PDT are important requirements for this treatment to become widespread in the clinics. When the mechanism of action of PDT is considered, the presence of molecular oxygen is required. Oxygen deficiency for various reasons may cause a decrease in the effectiveness of this treatment [37]. At the same time, the success of PDT depends on many parameters such as the chemical structure of photosensitizer, the properties of the light source, whether the area to be treated can receive enough light, the size and the anatomic structure of the region. All these features must be taken into account in PDT application [42]. Another disadvantage of PDT is that patients cannot implement this treatment by themselves as in antibiotic therapy. A specialist doctor and equipment are necessary to perform the treatment. However, with this treatment it is possible to eliminate the pathogens in patients after a few sessions [43]. Ongoing studies have led PDT to find a wide range of uses. Further studies are needed to establish a treatment protocol [41].

1.7 General Information About Fibroblast and Keratinocyte Cells

1.7.1 Fibroblast cells

The fibroblast cells, first described in the late 19th century, are mesodermal-derived cells in all connective tissues [44]. Fibroblast cells can be present in an active or inactive state. Active fibroblasts have a flattened star-shaped appearance and are generally found around collagen fibers. These cells have a large, an oval nucleus. When fibroblasts are in active state, ie during the growth process or during wound healing, an advanced golgi apparatus and granular endoplasmic reticulum can be observed in the electron microscope. Inactive fibroblast cells, also called fibrocyte, are smaller in size than the active ones and have a more oval shape. They have a smaller and darker painted nucleus. When examined by electron microscopy, they are seen that they have less granular endoplasmic reticulum [45].

Fibroblast cells have many physiological roles. These;

- In extracellular matrix synthesis, it is responsible for the production of collagen, glycaminoglycans and proteoglycans, which are the main constituents of extracellular matrix [46].
- Some fibroblast cells may differentiate into some cells in connective tissue, such as chondrocytes, osteocytes, adipocytes and smooth muscle cells.
- Fibroblasts are involved in the regulation of inflammation.
- There is a routine interaction between fibroblasts and immune system cells, and they help maintain homeostasis in mammalian tissues.
- Fibroblasts take a primary role in wound healing. The granulation tissue formed in the wound site contains a large amount of fibroblasts divided by the influence of various growth factors. Fibroblasts in the granulation tissue provide collagen production and provide resistance to the wound area [47, 48].
- Researchers use fibroblasts frequently in cell culture studies [44].

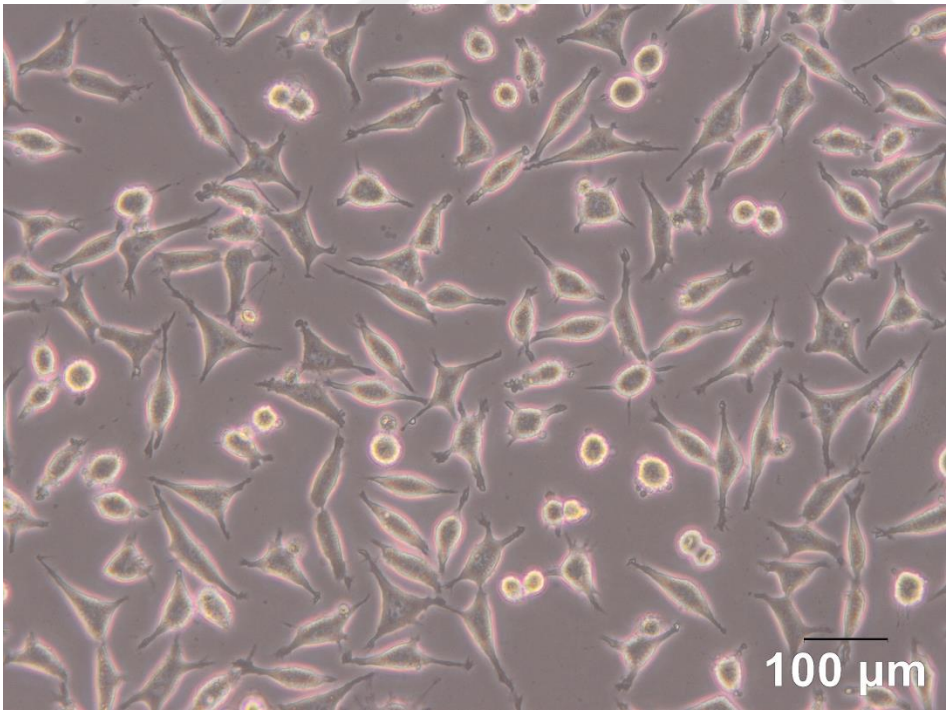

Figure 1.6 : Microscope image of fibroblast cells (magnification 20X).

1.7.2 Keratinocyte cells

The skin, which is considered as the largest organ of our body, has a very important function to separate the internal and external environment [49]. There are many cell types in the skin. The cells with the most function in the skin are known as keratinocytes, fibroblasts and melanocytes [50].

Keratinocytes are ectoderm-derived cells and allow the formation of epidermal structures in the body. For example; It is found in structures such as hair, nails, and sebaceous glands [50].

The main functions of keratinocytes are as follows:

- It forms the majority of the epidermis which is the outermost layer of the skin.
- It protects the body against external factors, acts as a barrier and prevents pathogens from entering the body [51].
- It contributes to wound healing. After injury, the immune cells increase and go towards the wound site. These cells are produced by epithelial keratinocytes [52].
- Keratinocytes provide the synthesis of some proteins such as involucrin, keratins, filaggrin, and transglutaminase [49].
- Keratinocytes have an important function in cell signaling in the extracellular matrix [51].

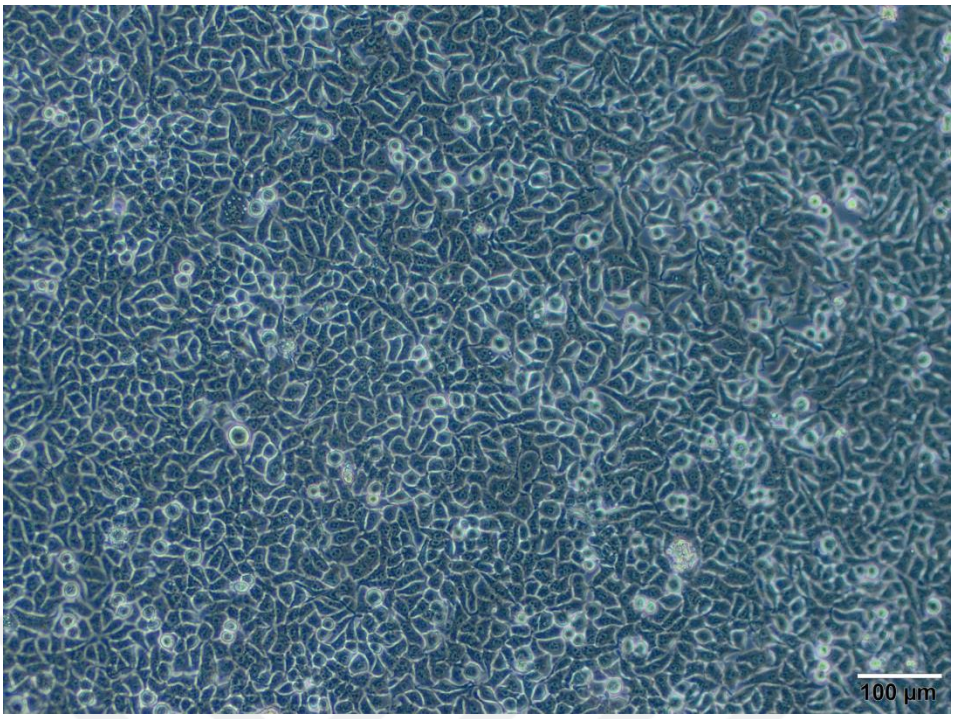

Figure 1.7 : Microscope image of keratinocyte cells (magnification 10X).

1.8 Possible Damages of PDT to Healthy Cells

As previously mentioned, photodynamic therapy is carried out by stimulating the photosensitizer applied in the target area with the appropriate wavelength of light. The molecules of photosensitizer cause the release of ROS by transmitting the excess energy which comes from light energy to the oxygen molecules in the environment. This causes apoptotic or necrotic cell death [9; 53].

When we look at the mechanism of cell death, it is important to determine where the photosensitizer is located in subcellular structures [54]. The photosensitizer may be accumulated in some parts of cells such as mitochondria, lysosomes, golgi apparatus, endoplasmic reticulum, cell membrane. In addition, the degree of hydrophobicity of the photosensitizer affects how the it is taken into the cell. The photosensitizers, which are hydrophobic in nature, enter the cell by spreading along the cell membrane and the less hydrophobic photosensitizers are taken into the cell by endocytosis. The photosensitizer uptaking by the cells is important in an effective PDT treatment because ROS has a short half-life and is effective only in its environment [7; 9]. Intravenously or topically applied photosensitizer accumulates selectively in the tumor or infected area [55]. Although photosensitizer mostly accumulates in the diseased tissue, there may be some photosensitizer accumulation in healthy cells of

surrounding tissue of the target. This leads damages to healthy cells too [56]. Cytotoxic products which are ROS released as a result of PDT lead to lipid peroxidation [57; 58]. Lipid peroxidation is the oxidative damage which occurs in lipids of cell membrane. These reactions cause structural and functional changes which are losing permeability and fluidity in cell membrane structure and losing activation of receptors on the membrane. At the end of these changes the cell death takes place [57; 59].

Although PDT has some side effects, good results are expected to be observed in the following post-treatment period. Therefore, a good understanding of this treatment mechanism and optimization is required. The development of new photosensitizers that can minimize the side effects and can be eliminated faster from healthy tissues is significant for the effectiveness of the treatment [7].

2. MATERIALS AND METHODS

In this study, the effect of antibacterial photodynamic therapy with indocyanine green on healthy mouse skin fibroblast cells and on healthy human keratinocyte cells was investigated *in vitro*. The experiments were performed in the Biomedical Optics and Laser Application Laboratory and Cleanroom and Cell Culture Laboratory of the Department of Biomedical Engineering in İzmir Katip Çelebi University.

There were 4 groups examined in this research:

1. Control group: Indocyanine green-free and laser-free group (C)
2. Indocyanine green group: Only indocyanine green applied group (ICG)
3. Laser group: Only laser applied group (L)
4. Photodynamic therapy group: Indocyanine green and laser applied group (PDT)

2.1 Cell Culture

Healthy mouse skin fibroblast cell line (L929) and healthy human skin keratinocyte cell line (HS2) were used in this study. These cell lines are in the Laboratories of the Department of Biomedical Engineering in İzmir Katip Çelebi University. First, these cells were proliferated in culture medium and prepared for photodynamic therapy applications. The procedure applied was the standard cultivation method. The cells were cultured in 25-cm^2 tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Aldrich) solution, containing 1% L-Glutamine (Gibco, USA), 10% Fetal Bovine Serum (FBS) (Gibco, USA) solution and 1% Penicillin/Streptomycin (Gibco,USA). These cells were incubated for 6 days in a humidified environment containing 95% air and 5% CO^2 until they form a confluent culture in a single layer. The growing medium were renewed every 2 days. When 80% confluence was reached, cells were washed with Phosphate Buffered Saline (PBS) (BioShop, Canada) solution and trypsinized using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Biological Industries, Israel). After 2-4 passages the cells were ready for the applications [12].

2.2 Light-Sensitive Substance

Indocyanine green, ICG (VWR, MP Biomedicals, USA), which has a higher absorption capacity for the wavelength of 808 nm (Figure 2.1, Figure 2.2), was used in this study as a photosensitizing agent. Before each experiment fresh solutions were prepared at specific concentrations which were determined for specific PDT applications. They were kept in darkness to prevent them from being affected by light. In this study, the effects of 4, 10, 25, 50, 100, 125 and 150 µg/ml ICG concentrations were examined on healthy cells by applying with or without specific energy doses of laser light as a combination for PDT applications which has shown the antibacterial effect on different bacterial strains in previous studies performed by Topaloglu and her colleagues (Figure 2.3) [60].

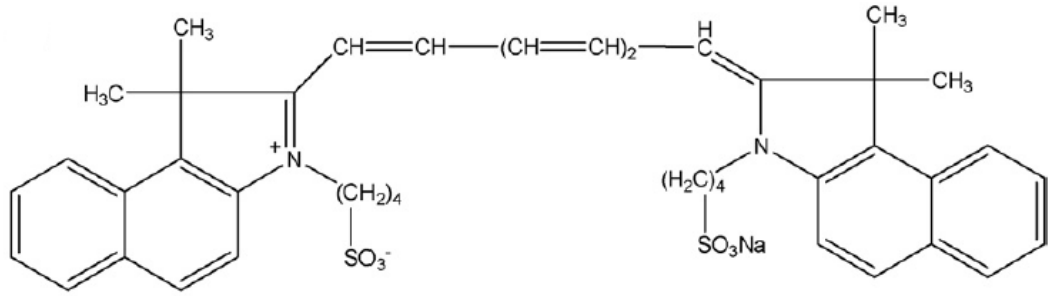

Figure 2.1 : Chemical structure of ICG [32].

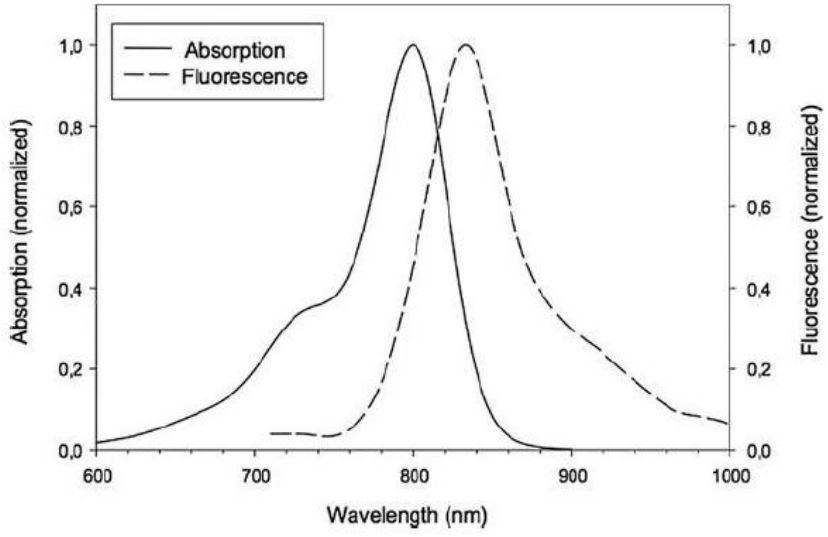

Figure 2.2 : Absorption spectrum of Indocyanine Green, Documents from Pulsion Medical System [61].

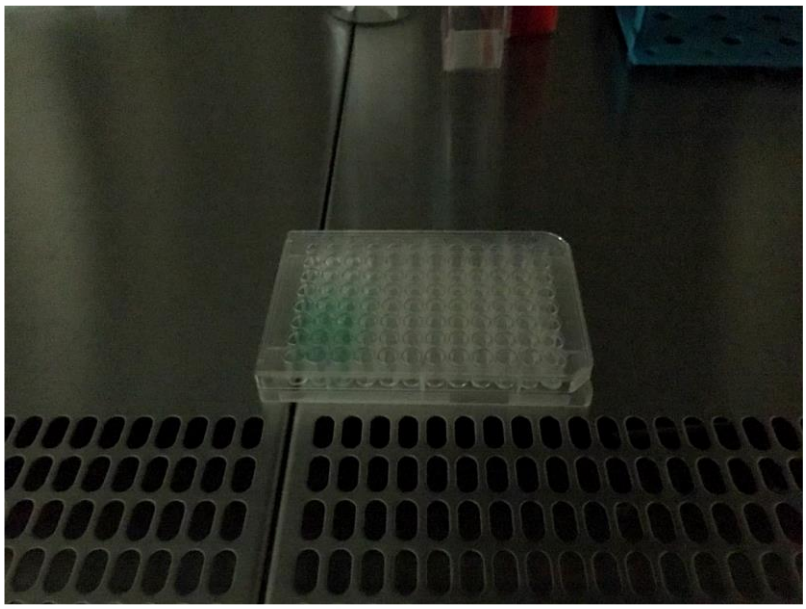

Figure 2.3 : Incubation of cells with ICG.

2.3 Laser System

In this study, a diode laser emitting at 808-nm of wavelength was used. This wavelength was chosen for being close to the highest value in the absorption band of ICG molecule. A diode laser which emits maximum 2 Watts of output power was used to induce the photosensitizer. This laser device (Teknofil, Istanbul, Turkey) were manufactured and purchased in accordance with experimental research (Figure 2.4). The cells in the Laser and PDT groups were exposed to energy densities of 84 and 252 J/cm^2, which have been determined as efficient energy dose to induce antibacterial PDT effect in previous studies (Figure 2.5) [60]. Prior to each experiment, the output power was controlled by using an optical power meter (THORLABS, Germany). The optical fiber of the laser system was fixed vertically on the optical table, emitting 1 W/cm^2 of power density on the surface of the well plate.

Figure 2.4 : Laser system.

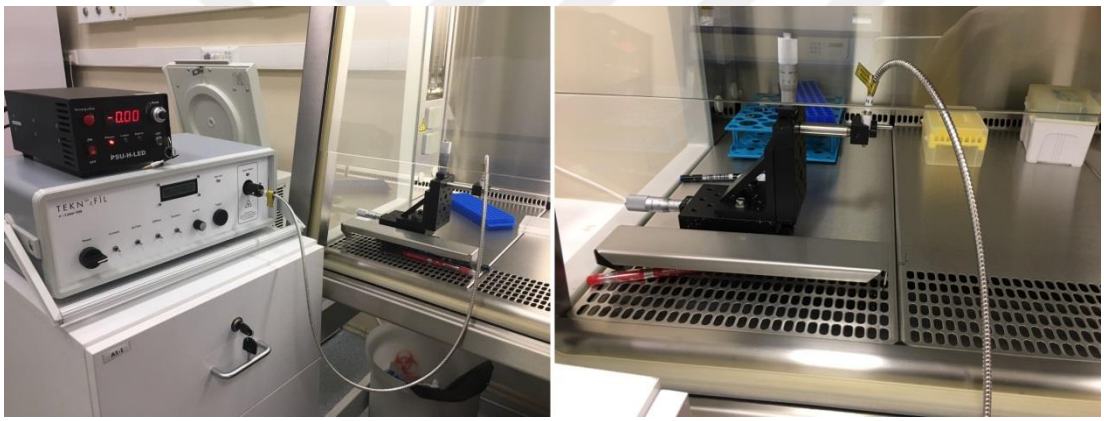

Figure 2.5: Use of the laser device in a laminar flow cabinet.

2.4 Absorption of ICG by Cells

This experiment was carried out to determine the amount of ICG taken into the fibroblast (L929) and keratinocyte (HS2) cells. For this; PBS solution, ICG dissolved in PBS solution and cells + ICG in PBS solution were prepared in 3 separate eppendorf tubes. ICG was prepared at 100 µg/ml concentration for L929 cells and ICG at 150 µg/ml concentration for HS2 cells. Because ICG has a higher absorption capacity for the wavelength of 808 nm, the absorption values at this wavelength were determined with UV-VIS Spectrophotometer.

The absorption value of ICG alone was determined based on PBS solution. Cells from eppendorf which contains cells incubated with ICG were eliminated and the absorption value of this solution was determined. The difference between the two absorbance values showed the amount of ICG taken into the cells.

2.5 Application of Photodynamic Therapy to The Cells

The cells were incubated overnight at 37 ºC for the adhesion to the surface of the plate after seeding them to 96-well plate with culture medium containing 10% FBS. Plates were prepared in 4 groups as control (C), only light (Laser), only drug (ICG) and experimental group (PDT) [73]. After 24 hours, groups of ICG and PDT were washed with PBS solution, 50 µl ICG solution in various concentrations was added to each well and then incubated at 37ºC for 15 minutes. In parallel, the growth medium in the Control and Laser groups were changed with fresh solutions and were incubated, photosensitizer was not added to these groups.

After incubation period, the cells in the Laser and PDT groups were illuminated with a specific energy density by an 808-nm diode laser device. Control and ICG groups were kept in the dark in meantime. After all applications were completed, the cells were ready for the analysis. All applications were performed at dark.

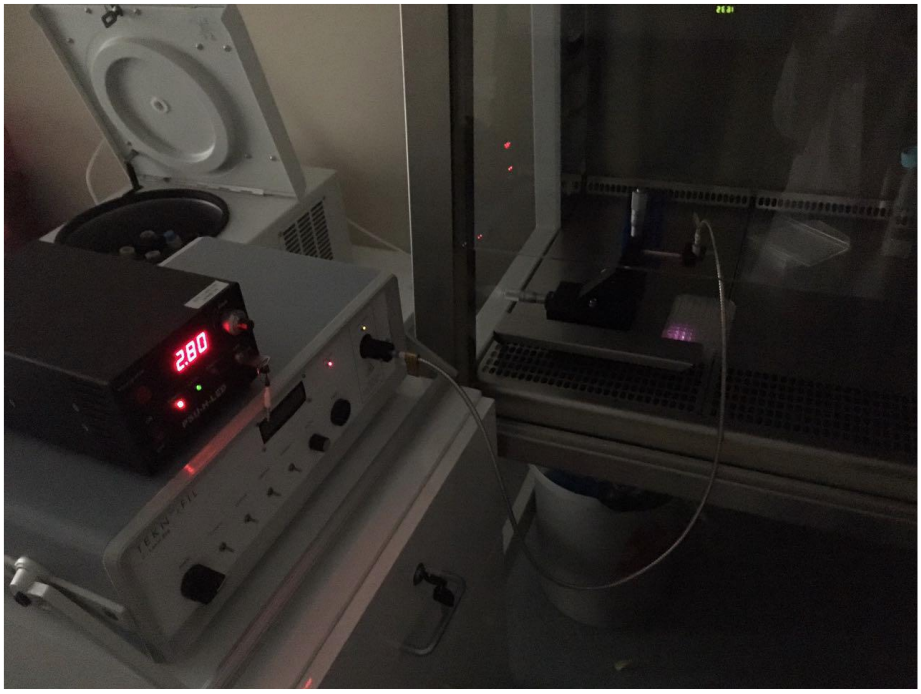

Figure 2.6 : Application of PDT in a dark environment in a laminar flow cabinet.

2.6 Cell Viability and Oxidative Stress Analysis After Applications

2.6.1 MTT analysis

After each application, the viability of the cells was calculated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma, Aldrich) analysis. The wells containing the cells were incubated at 37 ºC (5% CO_2) for 2 hours after newly prepared sterile 50 µl of MTT solution was added. Then MTT solution was removed from the wells and 50 µl of DMSO (Dimethyl Sulfoxide) (Merck, Germany) solution was added to each well. The plates were incubated for 5-10 min in shaker incubator. After incubation, absorbance values in each well were measured at 570 nm by Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). This density was an expression of the number of living cells in each well. The number of viable cells was calculated according to the control group and possible cytotoxic effects were determined. All applications were done at dark [68].

2.6.2 Lipid peroxidation (MDA) analysis

One of the most important targets of reactive oxygen species (ROS), the cytotoxic products of the photodynamic therapy mechanism, is lipid molecules in the cells. So MDA analysis method was used to measure the lipid peroxidation which may be the indicator of the produced ROS and magnitude of PDT application (MyBioSource, Lipid Peroxidation (MDA) Assay Kit, USA). After the applications, 50 µl of trypsin was added to the wells and incubated for 5 minutes. Then 150 µl of DMEM medium was added to the wells and the contents transferred to the centrifuge tube were centrifuged at 900 rpm for 5 min at 4°C. At the end of centrifugation, the supernatant in the tubes was discarded and 1 ml assay buffer was added to the pellet. The eppendorf tubes were held in the sonicator in turn (Figure 2.7) and then centrifuged at 8000 g at 4 ° C for 10 min. After centrifugation, 100 µl of supernatant and 200 µl of dye reagent were transferred to another eppendorf tube. The mixture was kept at 90°C for 30 minutes, then taken on ice. The centrifuge tubes were centrifuged at 1000 g 25°C for 10 min. 200 µl of supernatant was taken and transferred to the microplate. Absorbance values were measured at 532 and 600 nm with a microplate reader and the amount of lipid peroxidation was determined.

Figure 2.7 : Sonicator device.

2.6.3 Acridine orange / propidium iodide staining

This staining was performed to examine the effect of the applications on cell viability of the cells by fluorescence microscopy. The experimental solution was prepared by diluting the AO/PI stock solution (Logos Biosystems, USA) in the required ratio with PBS. For staining, medium was discarded from the cells in the wells and the cells were washed with sterile PBS. 50 µl of experimental solution was added to the cells and allowed to interact for 2 minutes in the incubator at 37 ºC. The staining solution was discarded and the cells were washed with PBS. The images of the cells were examined and visualized by fluorescence microscope (Olympus). These images were taken by CellSense Entry software and in these images live cells appear in green color and dead cells appear in red color.

3. RESULTS

3.1 The Effect aPDT Application on Mouse Skin Fibroblast Cells (L929)

3.1.1 Absorption of ICG by L929 cells

This examination was performed to determine the amount of ICG which is taken into the cells during applications. The absorbance value of the ICG solution which is the highest death rate of fibroblast cells at the 100 µg/ml concentration was measured at the spectrophotometer. The absorbance value of the solution containing cells incubated with ICG for 15 min was measured in order to determine how much ICG was introduced into the cells. The graph is shown in the Figure 3.1 according to the absorbance values obtained.

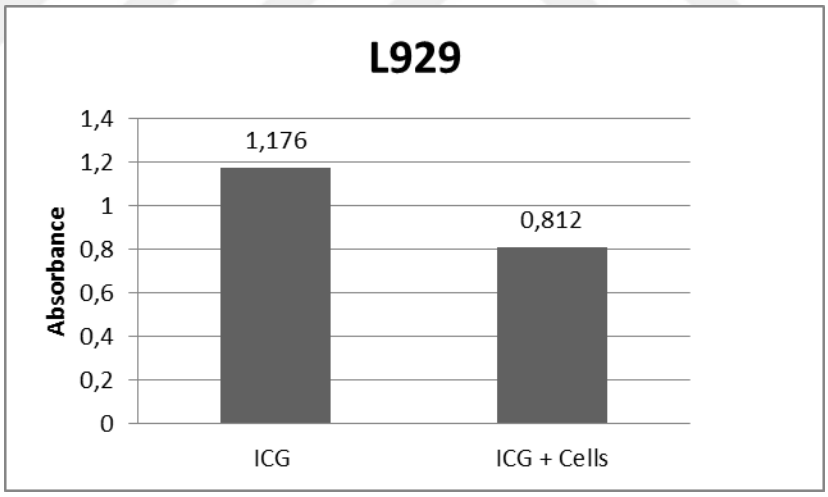

Figure 3.1 : Absorbance values of ICG and ICG + L929 Cells.

3.1.2 MTT analysis

To evaluate the cell viability; MTT (3 - [4,5-dimethylthiazol-2-yl] - 2,5 diphenyl tetrazolium bromide) analysis was performed after the applications of Control, Laser, ICG and PDT groups. Viable cell numbers were calculated according to the determined absorbance values. The treated groups were evaluated individually by comparing them with control group.

In the Laser group, Laser light was applied to the cells incubated in 96-well plates during an exposure time of 60 and 180 seconds for the energy doses of 84 J/cm^2 and 252 J/cm^2 respectively. As shown in the Figure 3.2, there was no significant difference in the viable cell count between Control and Laser groups as a result of statistical evaluation after laser applications with two different energy doses. It was determined that only Laser applications did not affect the cells in a positive or negative way for these energy doses.

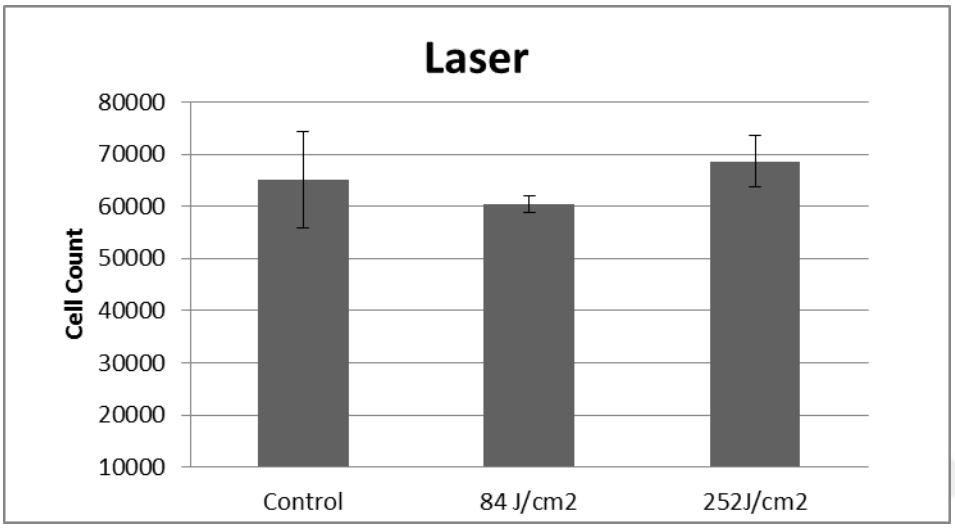

Figure 3.2 : Number of live cells after 84 and 252 J/cm^2 laser applications.

For the only ICG application, the cells were incubated with different ICG concentrations (4, 10, 25, 50, 100, 125 and 150 µg/ml). After incubation, MTT analysis was performed. Viable cell numbers were determined according to absorbance values and cell count for this group was compared with control group. As a result, the cells were not affected positively or negatively at 4 µg/ml. The ICG administered in the range of 10 - 150 µg/ml resulted in a significant amount of cell death (Figure 3.3). The maximum cell death rate was 53.87% at 150 µg/ml.

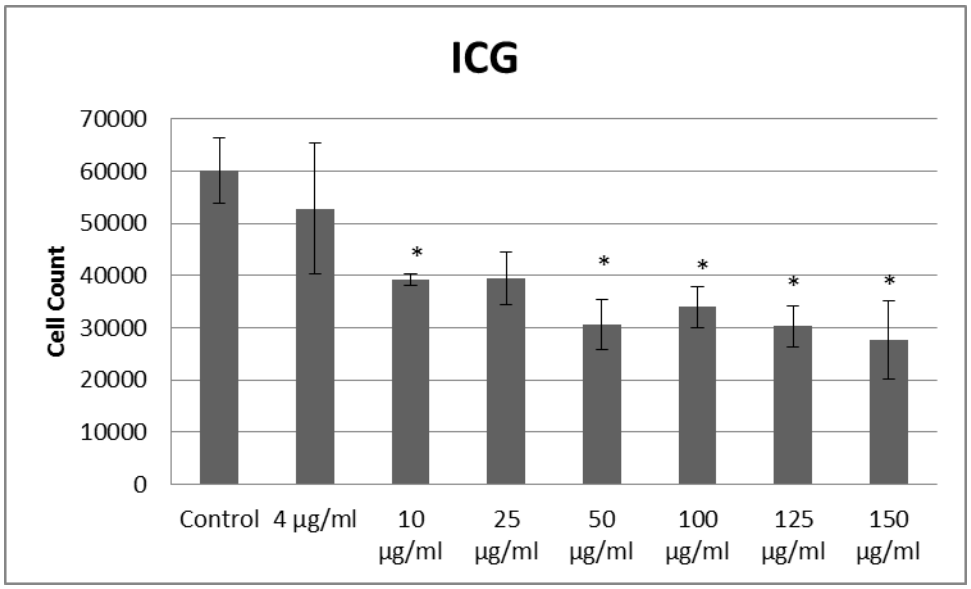

Figure 3.3 : Number of live cells after indocyanine green application at different concentrations. * indicates statistically significant difference between the experimental group and the control group ($p<0.05$).

To determine the effect of 84 J/cm^2 energy dose with different ICG concentration (4-150 µg/ml) on fibroblast cells, Laser light was exposed to the cells for 60 seconds after 15 minute incubation with ICG at certain concentrations. At the end cell viability was determined by MTT analysis.

After the PDT application with 84 J/cm^2 energy dose only 4 and 10 µg/ml ICG applications showed no statistically significant difference when compared with the control group. ICG concentrations in the range of 25-150 µg/ml resulted in significant cell death at a light dose of 84 J/cm^2 (Figure 3.4).

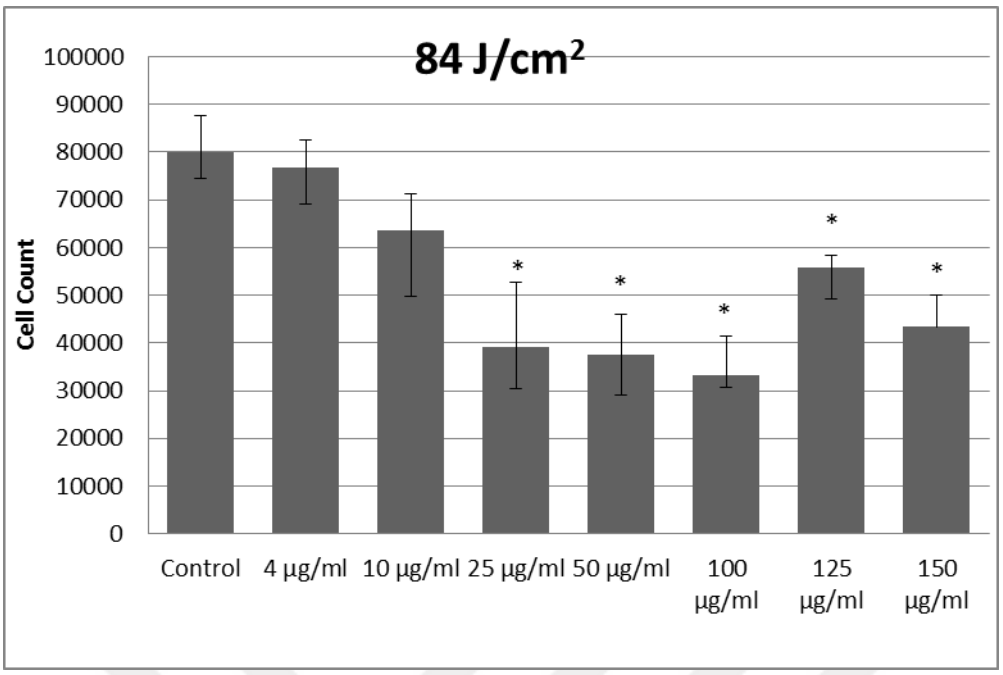

Figure 3.4 : Number of live cells after indocyanine green application at 84 J/cm^2 dose of energy and 4-150 µg/ml concentration. * indicates statistically significant difference between the experimental group and the control group ($p<0.05$).

For the PDT applications with the energy dose of 252 J/cm^2, cell were illuminated with laser light for 180 seconds and same ICG concentrations (in the range of 4-150 µg/ml) were applied. According to the statistical analysis, in the application of 252 J/cm^2 energy dose, 4 and 10 µg/ml ICG applications showed no statistically significant difference in the cells compared with the control group. As shown in Figure 3.5, statistically significant differences were observed in the range of 25-150 µg/ml when compared with control group (Figure 3.5).

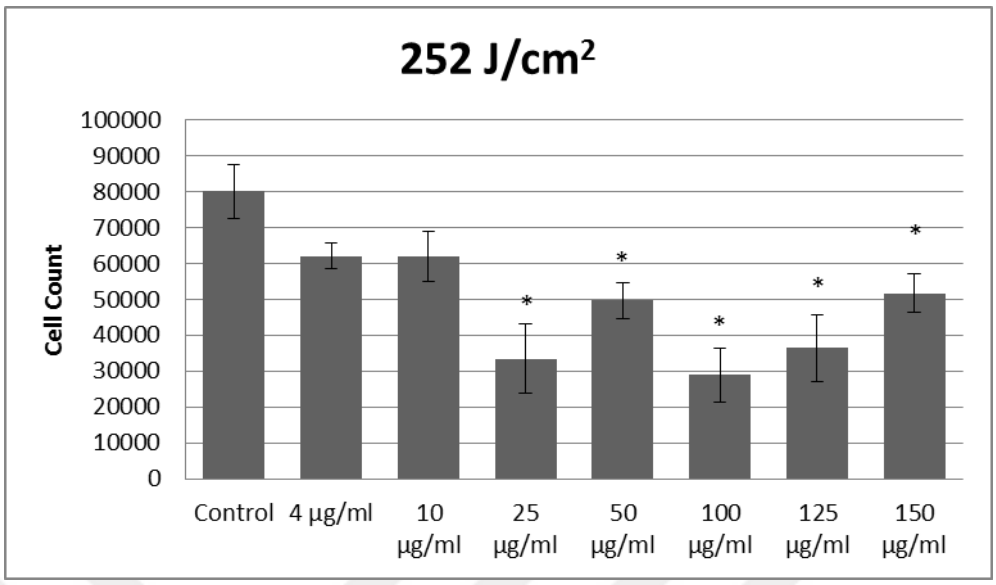

Figure 3.5 : The number of live cells after indocyanine green application at 252 J/cm^2 dose of energy and a concentration range of 4-150 µg/ml. * indicates statistically significant difference between the experimental group and the control group ($p<0.05$).

3.1.3 Lipid peroxidation (MDA) analysis

Cytotoxic products of photodynamic therapy are reactive oxygen species and they react with the lipid molecules in the cell membrane and inside the cell. This reaction cause irreversible damages in the cells. In this analysis method, the degradation products formed by the reaction of peroxide lipids with malondialdehyde (MDA) are determined by spectrophotometric method.

Figure 3.6 shows the amount of lipid peroxidation in cells treated with 84 and 252 J/cm^2 energy doses. There was no statistically significant difference between the groups.

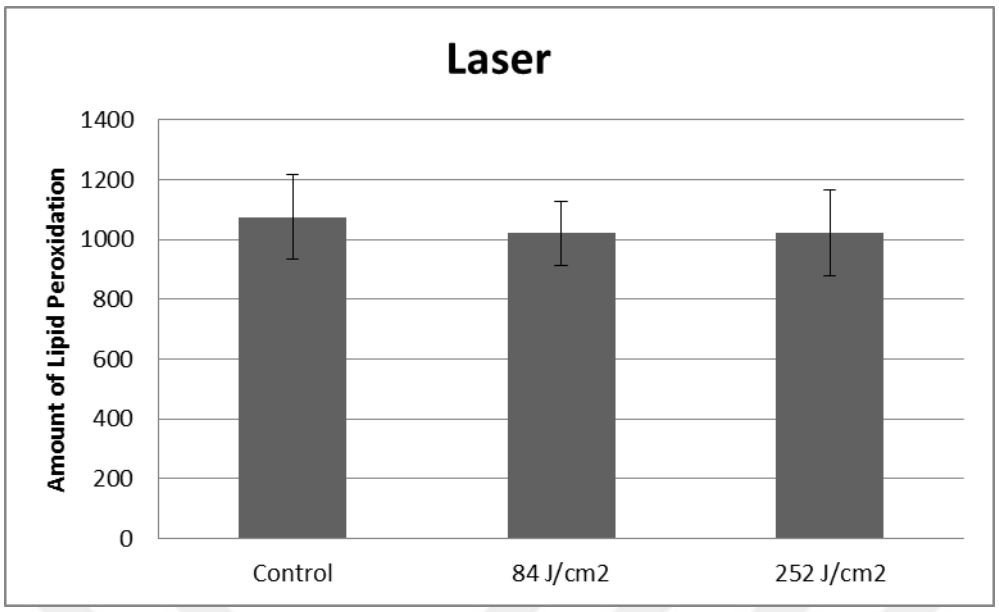

Figure 3.6 : Amount of lipid peroxidation caused by the application of 84 J/cm^2 and 252 J/cm^2 energy dose in the cells.

The amount of lipid peroxidation of 4, 25, 100 and 150 µg/ml ICG applied groups and ICG with the energy dose of 84 and 252 J/cm^2 caused by application is seen in the Figure 3.7. While the use of ICG alone was not statistically significant, the 25 µg/ml of ICG concentrations resulted in a significant difference in PDT groups with a dose of 84 J/cm^2. Although there was no statistically significant difference in PDT groups with an energy dose of 252 J/cm^2, the amount of lipid peroxidation was high compared to the other groups.

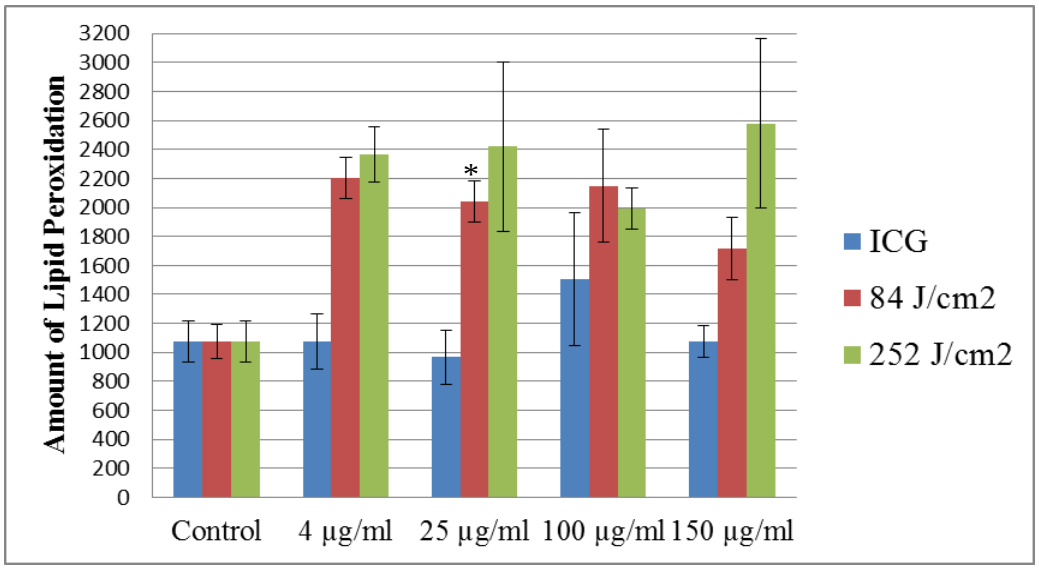

Figure 3.7 : Amount of lipid peroxidation in the cells caused by applications using 4, 25, 100, 150 µg/ml ICG and PDT groups which are applied 84 J/cm^2 and 252 J/cm^2 light doses. * indicates statistically significant difference between the experimental group and the control group ($p<0.05$).

3.1.4 Acridine orange / Propidium iodide staining

This procedure was performed to examine the effect of Photodynamic Therapy on cell viability by microscopy technique. Acridine orange and Propidium iodide dyes are nucleic acid-binding dyes. The AO dye stains live cells and provides green radiation and PI dye attaches to all dead cells and makes them red.

As shown in the Figure 3.8, the cells in the laser group treated with the 84 J/cm^2 and 252 J/cm^2 energy dose were similar to the control group and had no negative effect on the cell viability.

In the Figure 3.8, it is shown only ICG-applied cells at the second line of the image. There was an increase in the amount of dead cells as the drug concentration (concentration range of 4 – 150 µg/ml) increased in ICG treated groups only.

In the groups which are administered 84 J/cm^2 energy dose and ICG on fibroblast cells, while 4 µg/ml ICG did not show much negative effect on cells, the number of dead cells in other groups was quite high (Figure 3.8).

On the last line of the image (Figure 3.8) there are 252 J/cm^2 light dose and ICG applied PDT groups. Considering this light dose had a negative effect on all groups, it was observed that all cells died especially in 100 µg/ml and 150 µg/ml ICG groups.

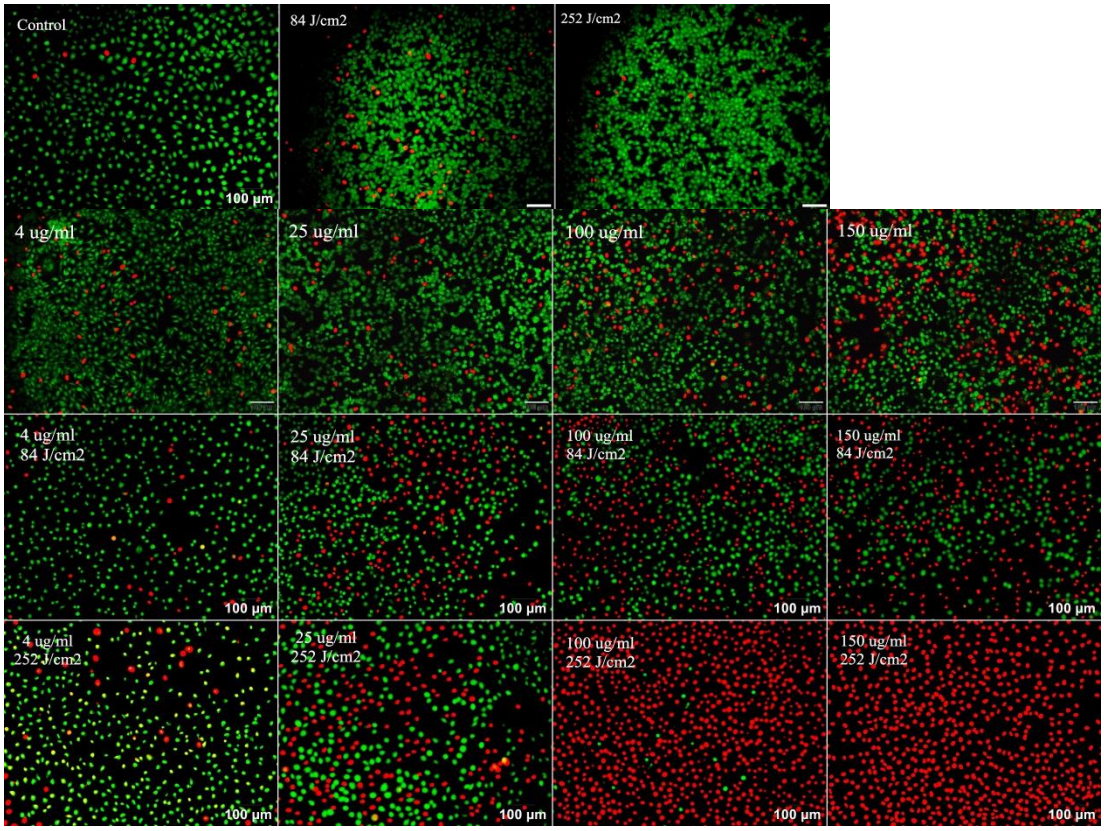

Figure 3.8 : Acridine Orange / Propidium Iodide Staining: Cell death and morphology caused by Laser, ICG, 84 J/cm^2 and 252 J/cm^2 PDT applications.

3.2 The Effect aPDT Application on Human Skin Keratinocyte Cells (HS2)

3.2.1 Absorption of ICG by HS2 cells

The absorbance value of the ICG solution which is the highest death rate of keratinocyte cells at the 150 µg/ml concentration was measured at the spectrophotometer. Absorbance values of the solutions containing cells incubated with ICG and only ICG were measured at 808 nm. It was found that the absorbance value of ICG was 2,260 and the absorbance value of ICG + Cells was 1,350. It was indicated the determined absorbance values in the Figure 3.9.

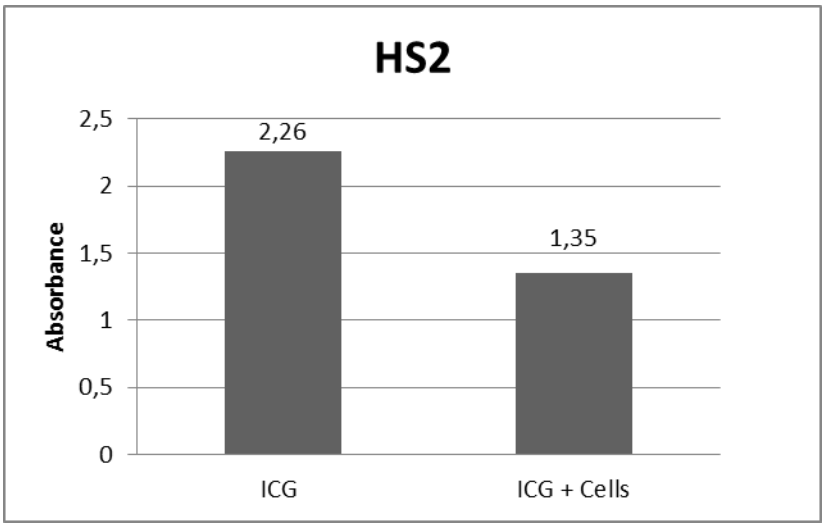

Figure 3.9 : Absorbance values of ICG and ICG + HS2 Cells.

3.2.2 MTT analysis

To examine the effect of the laser on HS2 cells, 84 J/cm^2 and 252 J/cm^2 energy doses were applied. Viable cell numbers were determined according to absorbance values obtained from MTT analysis. As shown in the Figure 3.10, the laser applied at two different energy doses did not have a positive or negative effect on cell viability.

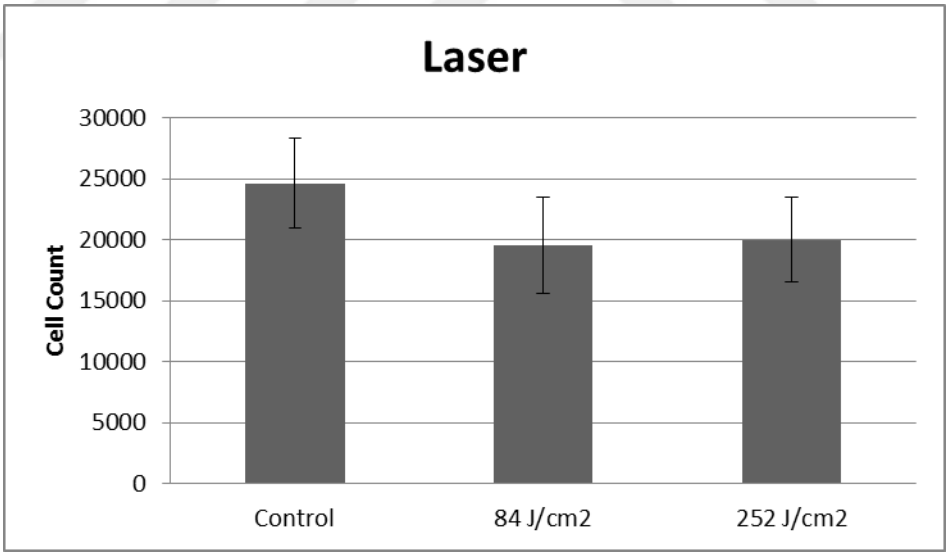

Figure 3.10 : Live cell count after 84 and 252 J/cm^2 laser applications.

To determine how keratinocyte cells were affected only by photosensitizer application, ICG was applied to the HS2 cells with solutions in the concentration range of 4-150 µg/ml. After the applications, absorbance values were determined by MTT analysis and live cell numbers were calculated (Figure 3.11).

According to the results of the statistical evaluation made between the control and the experimental groups, the drug administered in the concentration range of 4-50 µg/ml did not have a positive or negative effect on the cells. In the groups with 100-150 µg/ml ICG concentration, statistically significant cell death was observed when compared to the control group.

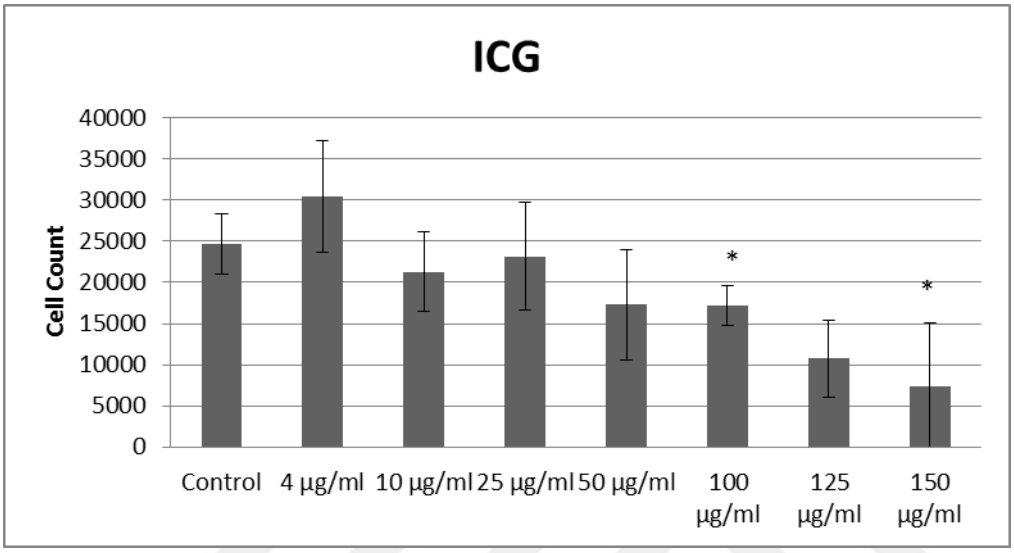

Figure 3.11 : Number of live cells after indocyanine green application at different concentrations.

Cell viability was determined by the MTT analysis method in the photodynamic therapy groups at which the drug combination was administered at certain energy doses (84 J/cm^2 and 252 J/cm^2) and at certain concentrations (4, 10, 25, 50, 100, 125, 150 µg/ml).

According to the MTT analysis performed after the PDT applications with 84 J/cm^2 of energy dose and different ICG concentrations (in the range of 4-150 µg/ml) on HS2 cells, no statistically significant cell death was observed on the cells which received 84 J/cm^2 energy dose and ICG concentration of 4-125 µg/ml. A statistically significant cell death was observed in the group treated with 150 µg/ml compared to the control group. It has been determined that the use of 150 µg/ml of ICG concentration on healthy cells causes death by damaging them (Figure 3.12).

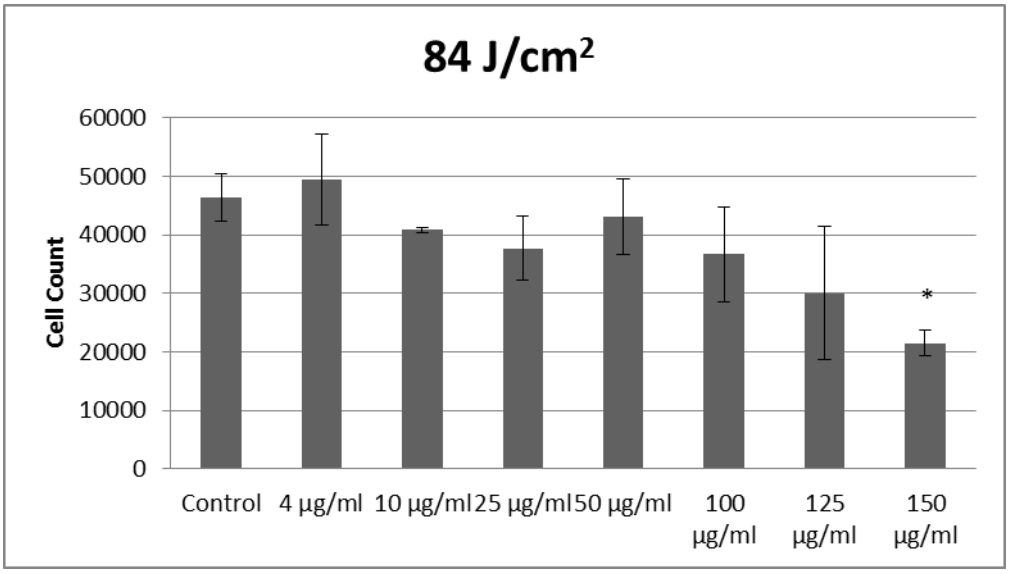

Figure 3.12 : Number of live cells after indocyanine green application at 84 J/cm^2 energy dose and 4-150 µg/ml concentration. * shows that there is a statistically significant difference between the experimental group and the control group ($p<0.05$).

Cell viability was assessed by performing MTT analysis in photodynamic therapy groups with the combination of 4-150 µg/ml ICG concentration and 252 J/cm^2 energy dose. A statistical evaluation was made by comparing with control group and cell numbers determined according to absorbance values. According to these results, statistically significant cell death was observed in all drug concentrations. This application with 252 J/cm^2 has been defined as harmful to keratinocyte cells (Figure 3.13).

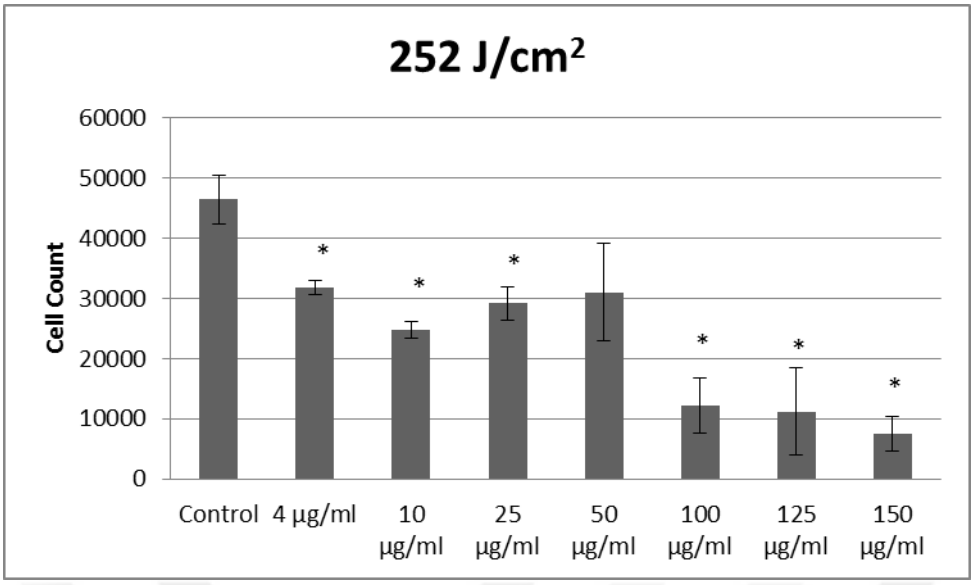

Figure 3.13 : Number of live cells after indocyanine green application at 252 J/cm^2 energy dose and in a concentration range of 4-150 µg/ml. * shows that there is a statistically significant difference between the experimental group and the control group ($p < 0.05$).

3.2.3 Lipid peroxidation (MDA) analysis

Cytotoxic products resulting from the Photodynamic Therapy mechanism cause lipid peroxidation by interacting with lipid molecules in the cell membrane and inside the cell. To detect this, lipid peroxidation (MDA) analysis was made after the applications with keratinocyte cells.

The amount of lipid peroxidation in the cells as a result of laser application at two different energy doses (84 J/cm^2 and 252 J/cm^2) is shown in the Figure 3.14. According to the results, the amount of lipid peroxidation did not differ from the control group in the administration of both of the two energy doses (Figure 3.14).

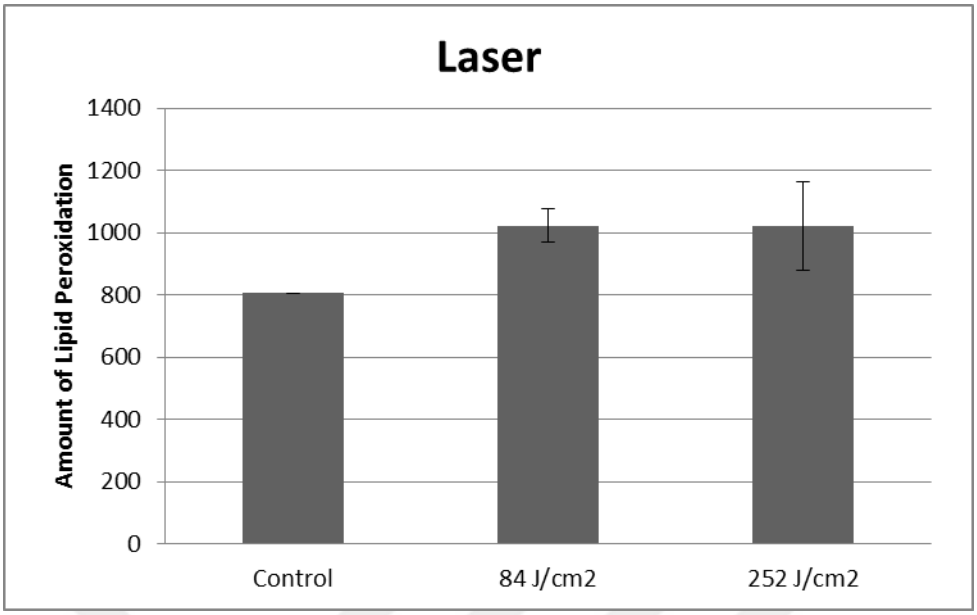

Figure 3.14 : Lipid peroxidation amount after 84 J/cm^2 and 252 J/cm^2 laser applications.

The amounts of lipid peroxidation caused by ICG groups and PDT groups which were used two different energy doses (84 J/cm^2 and 252 J/cm^2) are shown in the Figure 3.15. According to statistical analysis, it was observed significant difference in the 252 J/cm^2 energy dose and 125 µg/ml ICG applied group only. Although there was no statistically significant difference in the other groups, the amount of lipid peroxidation in all groups was higher compared with the control group.

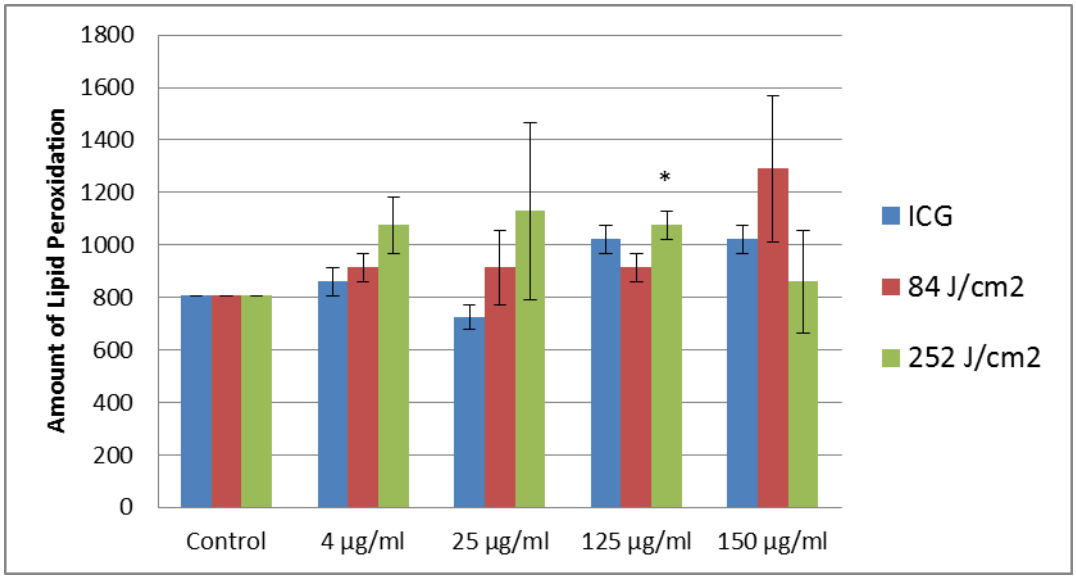

Figure 3.15 : Amount of lipid peroxidation in the cells caused by applications using 4, 25, 125, 150 µg/ml ICG and PDT groups which are applied 84 J/cm^2 and 252 J/cm^2 light doses. * indicates statistically significant difference between the experimental group and the control group ($p<0.05$).

3.2.4 Acridine orange / Propidium iodide staining

Acridine Orange / Propidium Iodide Staining analysis method which performed by using AO and PI dyes was used to observe live and dead cells by fluorescence microscope after the ICG, Laser and PDT applications. With this analysis live cells appear in green color and dead cells appear in red color. Thus, they can be easily distinguished from each other.

The images of cells received 84 J/cm^2 and 252 J/cm^2 energy doses and the cells in the control groups are presented below (Figure 3.16). As seen in the images, laser application alone did not have a negative effect on the cells and similar images were obtained as in the control group.

As shown in the Figure 3.16, no significant cell death was observed in the cells only in the ICG group compared to control group. In parallel with the MTT analysis results, as the drug concentration increases, more red stained cells was observed.

In the group which 84 J/cm^2 energy doses are administered to keratinocyte cells with 4 different drug concentrations, as the amount of the drug increased, a linear increase in the rate of cell death was observed (Figure 3.16). When the cell death in PDT groups which are using 4 and 25 μg/ml ICG compared with the control group it can be seen that the number of red cells is similar. However, the number of red cells was quite high in the groups where 125 and 150 μg/ml ICG was used.

In the application of 252 J/cm^2 energy dose, cell death increased linearly as the drug concentration increased. It was found that 125 and 150 μg/ml drug concentration and 252 J/cm^2 energy dose application killed all of the cells. All drug concentrations showed a significant difference in the cell death rate compared to the control group (Figure 3.16).

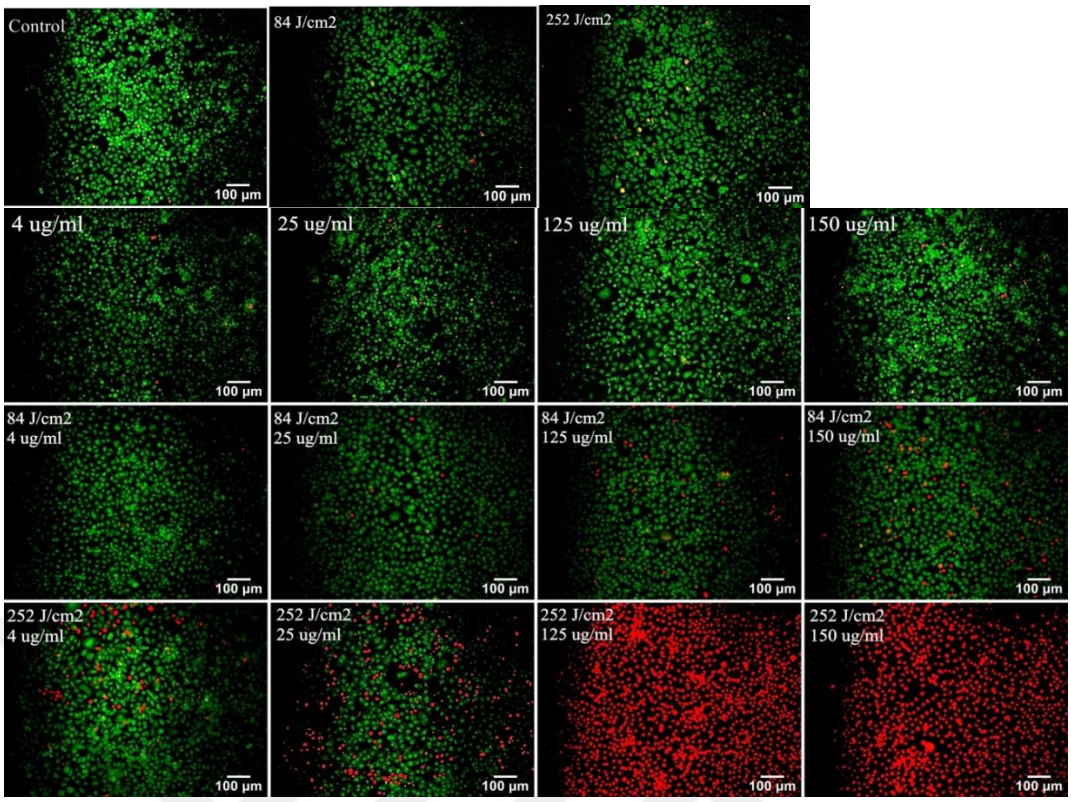

Figure 3.16 : Acridine Orange / Propidium Iodide Staining: Cell death and morphology caused by Laser, ICG, 84 J/cm^2 and 252 J/cm^2 PDT applications.

4. DISCUSSION

Photodynamic therapy is a treatment method in the presence of light-sensitive substance (photosensitizer), light in the appropriate wavelength that can stimulate the photosensitizer and oxygen in the environment. As an alternative method to treat infection, antibacterial photodynamic therapy is a highly emphasized issue in recent years. Especially in the antibiotics treatment, which is the classical treatment method, due to resistance development of bacteria and treatment of disease has become more difficult, more importance has given to antibacterial photodynamic therapy [62]. In order to kill different pathogens, many photosensitizers and light sources are investigated in this treatment technique.

ROS which is produced due to the mechanism of antibacterial photodynamic therapy, interacts with bacterial cells or structures within the cell, ensures the killing of bacteria. The amount of light and photosensitizer used varies depending on the type of bacteria desired to be killed. Gram-negative and gram-positive bacteria have different cell wall structure. Gram-positive bacteria have a thick layer of peptidoglycan on the cell membrane, while gram-negative bacteria contain a thin layer of peptidoglycan and an outer membrane on the cell membrane [28] (Figure 4.1). In this respect, gram-negative and gram-positive bacteria are affected differently during antibacterial photodynamic therapy [62]. Antibacterial photodynamic therapy is more effective on gram-positive bacteria because it is easier to cross the cell wall consisting of peptidoglycan for photosensitizer and interact with the cytoplasmic membrane. Anionic or neutral photosensitizers bind to gram-positive bacteria easily and provide photoinactivation, while the outer membrane in gram-negative bacteria makes photoinactivation difficult [38; 40]. In this regard, more light and drug doses should be used to treat infections caused by gram-negative bacteria [61].

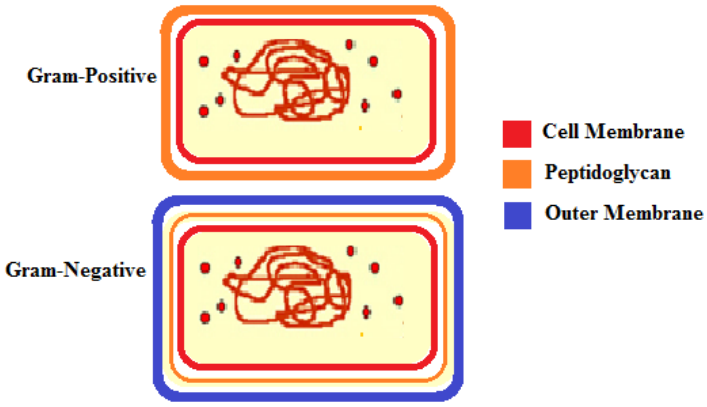

Figure 4.1 : The figure represents the cell wall of gram positive and gram negative bacteria.

Another important issue to be considered when examining antibacterial photodynamic therapy in the treatment of infection is how healthy cells in the environment are affected by this treatment method. It is important to investigate the cytotoxic effects caused by aPDT in cells such as fibroblast and keratinocytes, which have important functions in the healing process of wound infections [63; 64]. In this study, cytotoxic effects of PDT on healthy fibroblast and keratinocyte cells were examined using different ICG concentrations and light doses with previously proven antibacterial activity.

4.1 Applications on Mouse Skin Fibroblast Cells (L929)

Firstly, antibacterial-proven doses were applied on healthy fibroblast cells and cell viability was determined by MTT analysis. According to the results of the MTT analysis, it was found that the light dose of 84 J/cm^2 and 252 J/cm^2 did not have a negative effect on the cells, whereas in ICG applications only with 4 µg/ml and 25 µg/ml did not cause any negative effect on the cells. While 10 µg/ml ICG alone had negative effects on cells, this concentration was not statistically different in 84 J/cm^2 and 252 J/cm^2 PDT applications and did not significantly affect the cells. Low doses of light are known to cause cell proliferation by triggering some biochemical pathways in the cells [65]. With this mechanism which is biostimulation, it is possible that the concentration of 10 µg/ml in the PDT groups does not damage the cells.

In the PDT groups with light doses of 84 J/cm^2 and 252 J/cm^2 and 4-150 µg/ml ICG concentrations, 4–10 µg/ml ICG did not show any harmful effects on L929 cells. Severe cell deaths were observed in PDT groups with 25-150 µg/ml ICG. In the study of Topaloğlu et al. (2013), it was observed that the energy dose of 84 J/cm^2 with 4-6 µg/ml ICG concentration which are effective in killing *S.aureus* [60] did not damage the fibroblast cells. In the PDT applications with 252 J/cm^2 energy dose and 100-125 µg/ml of ICG concentrations which are effective in destroying the *P.aeruginosa* strain [60], the cells were severely damaged.

Lambrechts et al. have identified lethal doses according to their previous antimicrobial photodynamic therapy study on pathogens of *S.aureus, P.aeruginosa* and *C.candida,* which are involved in wound infections. Based on the doses they used, they conducted a study to evaluate the cytotoxicity on healthy dermal fibroblast cells. In this study, they used TriP [4] as a photosensitizer. At doses where 99,99% of *S.aureus* was killed, fibroblast viability decreased by 78,9%. At the dose of photosensitizer where 92,12% of *P.aeruginosa* was killed, fibroblasts died at a rate of 94,7%. *S.aureus* could be killed by using similar lower doses like at the referenced doses which are used in this study [60], while higher doses were needed to kill *P.aeruginosa*. This showed that doses in which gram positive bacteria could be inactivated resulted in less harmful effects on fibroblasts [63**].**

Ribeiro et al. in their study, they used curcimin as a photosensitizer and LED as a light source. They compared using different light doses and drug concentrations on the MRSA and L929 fibroblast cell line. While 100% photodynamic inactivation of MRSA was observed, 80% reduction in fibroblast metabolism was observed and they concluded that PDT was more effective against bacteria [66].

In the study of Gomes-Filho et al. performed in 2016, they reported that aPDT with curcimin and 480 nm wavelength of light decreased the viability of mouse fibroblast cells (L929) by less than 25%. The drug concentration they used was 500 mg/L and the light dose was 72 J/cm^2. These parameters are less than the doses used in this study, so the amount of affected fibroblast cells were less than the amount of affected fibroblast cells in this study too [67].

In 2013, Kashef et al. obtained antibacterial effect on *S. aureus, E. faecalis and E. coli* pathogens in low hypericin concentrations while fibroblast cells were not damaged. However, these parameters were not sufficient to kill *P.aeruginosa* which is a gram negative bacteria [68].

According to the results obtained in Lipid Peroxidation (MDA) analysis, only 84 J/cm^2 energy dose and 25 µg/ml ICG administered PDT group was statistically different compared with the control group. Though statistical analysis gave this result, a greater amount of lipid peroxidation was observed in all PDT groups when compared with the control group. Considering MTT results, consistent results were not generally observed when the reduction in cell viability and lipid peroxidation analysis were compared. When we evaluated PDT groups among themselves, we found more consistent results. As seen in the Table 4.1, the amount of lipid peroxidation was lower in ICG and laser groups while it was higher in PDT groups. The amount of lipid peroxidation that occurs in 4 µg/ml ICG treated PDT groups was higher than the ICG applied PDT groups with higher concentrations (Table 4.1), this may be the result of the onset and rapid completion of lipid peroxidation reactions caused by ROS produced in the cell. Apart from this, the reason for the lower amount of lipid peroxidation than the amount we expected was that some of the ICG was taken in the cell and therefore the reactions started there. In our experiment to determine the amount of ICG taken into the cell, 100 µg/ml ICG solution gave an absorbance value of 1,176, while the solution containing cells incubated with the same amount of ICG gave an absorbance value of 0,812. This expressed that ICG was taken into the cell. In this respect, the lipid peroxidation incident that occurs in the lipids of the organelles inside the cell and if the chain reactions occur rapidly, the reaction can be completed until it reaches the cell membrane, perhaps it can reach enough saturation and the resulting lipid peroxidation may decrease later on.

Table 4.1 : Percentage changes in MTT and Lipid Peroxidation Analysis with respect to the control group in the applications of Laser, ICG and PDT groups.

L929 Cells

Applications	MTT (%)	Lipid Peroxidation (%)
84 J/cm^2	-7,23	-5
252 J/cm^2	+5,52	-5
4 µg/ml	-12,11	0
25 µg/ml	-34,52	-10
100 µg/ml	-43,58	+40
150 µg/ml	-53,87	0
84 J/cm^2 + 4 µg/ml	-4,07	+105
84 J/cm^2 + 25 µg/ml	-51,22	+90
84 J/cm^2 + 100 µg/ml	-58,39	+100
84 J/cm^2 + 150 µg/ml	-45,76	+60
252 J/cm^2 + 4 µg/ml	-22,49	+120
252 J/cm^2 + 25 µg/ml	-58,20	+125
252 J/cm^2 + 100 µg/ml	-63,94	+85
252 J/cm^2 + 150 µg/ml	- 35,44	+140

Acridine orange / propidium iodide staining was performed to observe the viability of the cells. The obtained images supported MTT results. As the drug concentration and the dose of light increased, the amount of dead cells increased. 100 µg/ml ve 150 µg/ml ICG caused higher amount of cell death.

4.2 Applications on Human Skin Keratinocyte Cells (HS2)

Light and ICG doses which are proven to have antibacterial activity [60] were applied on keratinocyte cells, too. MTT analysis was performed to determine cell viability. According to the results of the analysis, it was determined that 84 J/cm^2 and 252 J/cm^2 light application had no negative effects on the cells. The cells in the ICG group with 100 µg/ml and 150 µg/ml were significantly affected negatively. In this respect, it can be said that keratinocyte cells are more resistant to ICG than fibroblasts. In the PDT group with 84 J/cm^2 of light dose, a statistically significant difference was observed in only 150 µg/ml ICG. Except for 50 µg/ml ICG group, severe cell death occurred in all PDT groups with a light dose of 252 J/cm^2.

The reason for this may be that the lethal effect is suppressed due to the proliferation effect of light. Topaloğlu et al. (2015) [65] and Karam et al. (2017) [69] showed possible biostimulation effects of aPDT in their studies.

Jancˇula et al. (2013) investigated the effects of different phthalocyanine species on human keratinocyte cells (HaCaT). They used a white LED lamp as a light source. Similarly to the results of this study, it was observed minimal cytotoxicity effects on the cells in the test groups where they did not apply light, while light + drug combination showed significant cytotoxic effects in keratinocyte cells [70].

In 2011, Maduray et al. investigated the effect of PDT on melanoma, fibroblast and keratinocyte cells. At 50 µg/ml concentration of ZnTSPc and 4.5 J/cm^2 light dose (672 nm) decreased cell viability to 61% in melanoma cells, while it decreased to 81% and 83% in fibroblast and keratinocyte cells, respectively. The decrease in keratinocyte cells was nearly the same with the result that we obtained at a concentration of 84 J/cm^2 and 25 µg/ml ICG applied to keratinocyte cells (18.81% decrease). In this study, 20% cell viability decrease in fibroblast cells in the 84 J/cm^2 + 10 µg/ml PDT group was similar to the results in the study of Maduray et al.. In this regard, lower drug concentration and higher energy dose have shown similar effects [71].

Kashef et al. (2012) investigated the effects of PDT using methylene blue (MB) and toluidine blue (TBO) and a red light source on fibroblast cells. The viability of fibroblast cells decreased by 27 % (MB) and 39.6 % (TBO) at doses which MRSA died 99% determined in their previous studies [72].

In 2017, Gharesi et al. performed PDT on human gingival fibroblast cells. In their study, they used ICG as a photosensitizer and 808 nm diode laser as a light source. They reported that the expression of the BAX gene, which is a gene associated with apoptosis, was induced after 1000 µg/ml concentration and 39 J/cm^2 light dose administration. Only laser and ICG applications did not cause significant apoptotic effect. Laser application did not cause negative effects on the cells in this study, too [73].

Pourhajibagher et al. examined the effect of aPDT which is an alternative treatment for the treatment of dental infections, using ICG (in the range of 500 - 2000 µg/ml) and an 810 nm wavelength diode laser, on human gingival fibroblast cells (HuGu) in 2016. They noticed that the cell viability decreased, when they increased the duration of the laser application and decreased ICG concentration.,They reported a significant decrease in cell viability at or below 1000 µg/ml ICG. In their study, they used much more ICG than the ICG concentrations used in this study [12].

To determine the amount of ICG that taken into keratinocyte cells, the absorbance values of the 150 µg/ml of ICG solution and solution which contains the cells incubated with the same amount of ICG were measured by spectrophotometer. Accordingly, it was found that the absorbance value of ICG was 2,260 and the absorbance value of ICG + Cells was 1,350. These values have proven that ICG accumulate within the cells. In this respect, similar to the results obtained in fibroblast cells, a small amount of lipid peroxidation may suggest that lipid peroxidation reactions occur inside the cell and may result in lesser amounts. Lipid peroxidation amounts were statistically significant only in the 252 J/cm^2 + 125 µg/ml PDT group but overall they were all higher than the control group. The results of MTT analysis and lipid peroxidation analysis in PDT groups were generally consistent (Table 4.2). However, in the 252 J/cm^2 + 150 µg/ml PDT group which had highest death rate, amount of lipid peroxidation was low compared to expected (Table 4.2).

Table 4.2 : Percentage changes in MTT and Lipid Peroxidation Analysis with respect to the control group in the applications with Laser, ICG and PDT groups.

HS2 Cells

Applications	MTT (%)	Lipid Peroxidation (%)
84 J/cm^2	-20,63	+26,67
252 J/cm^2	-18,82	+26,67
4 µg/ml	+23,78	+6,67
25 µg/ml	-5,92	-10,00
125 µg/ml	-56,36	+26,67
150 µg/ml	-70,11	+26,67
84 J/cm^2 + 4 µg/ml	+6,69	+13,33
84 J/cm^2 + 25 µg/ml	-18,81	+13,33
84 J/cm^2 + 125 µg/ml	-35,40	+13,33
84 J/cm^2 + 150 µg/ml	-53,65	+60,00
252 J/cm^2 + 4 µg/ml	-31,54	+33,33
252 J/cm^2 + 25 µg/ml	-37,17	+40,00
252 J/cm^2 + 125 µg/ml	-75,82	+33,33
252 J/cm^2 + 150 µg/ml	-83,73	+6,67

Cell viability with AO / PI staining analysis was observed by microscope. Cell death was observed in a similar manner with the results of MTT analysis. There was an increase in the number of red stained cells which are dead cells by dose increase. 252 J/cm^2 of light dose 125 µg/ml and 150 µg/ml of ICG killed all of the cells by showing serious cytotoxic effects.

In this study, it was determined that PDT can be applied until 25 µg/ml ICG concentrations without harming fibroblast cells by using 84 J/cm^2 and 252 J/cm^2 of energy dose. When the same doses were used in keratinocyte cells, the cells up to 125 µg/ml ICG at 84 J/cm^2 of energy dose in PDT were not damaged, but all ICG concentrations at 252 J/cm^2 were found to be detrimental. Use of these doses in the clinic can cause serious side effects and harm patients. For this reason, it is necessary to minimize the harmful effects of antibacterial PDT application on the cells by dose adjustment. Safe dose ranges should be determined and applications should be performed.

5. CONCLUSION

The development of resistance to many antibiotics by bacteria which cause wound infections generates a threat to human and animal health worldwide. In recent years, it has been begun to focus on antibacterial photodynamic therapy as an alternative treatment method. While in this treatment it is intended to kill a high percentage of pathogens, another important issue to be considered is how healthy cells around the infection site are affected.

In this study, the effects of antibacterial photodynamic therapy were examined on fibroblast and keratinocyte cells which have important functions in wound healing. As a result of the these applications, it was found that healthy skin fibroblast and healthy skin keratinocyte cells were not damaged at photosensitizer concentration and light dose which is used to kill gram positive *S. aureus*. On the other hand, these cells were severely damaged at photosensitizer concentration and light dose which achieve photoinactivation of gram negative bacteria *P. aeruginosa*.

According to this study, it was concluded that photodynamic therapy applications which destroy pathogens, may kill healthy eukaryotic cells. While some doses are safe, some doses cause serious death of healthy fibroblasts and keratinocyte cells. Therefore, harm to healthy cells in photodynamic therapy should be minimized. The optimization of light dose and drug concentration should be done properly. Dose optimization should be provided by knowing the light dose and drug concentration that cause eukaryotic cell death. In cases which may cause eukaryotic cell death, different doses or treatment methods should be applied. If high doses need to be used, damage to healthy cells should be minimized by using methods such as targeted therapy.

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Kadikoylu G., Onak G., Karaman O., Topaloglu N., (2018) "How Does Antibacterial Photodynamic Therapy Affect Healthy Human Keratinocyte Cells?" TIPTEKNO'18, 8–10 November, Gazi Magosa, KKTC (Oral Presentation)

Gur N., **Kadikoylu G.,** Ozdemir F. A., Topdemir A., Duzel A., (2015) "In Vitro Bulblet Regeneration from *Pancratium maritimum* Twin Scale Explants" VIIth International Bioengineering Congress, 19 - 21 November, Izmir, Turkey. (Poster Presentation)

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