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Possible Biostimulative Effect of Low-Dose Photodynamic Therapy on HUVEC Cells

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by

Dilara Portakal Koç

ORCID 0000-0001-8043-712X

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APPROVED BY:

Advisor: **Dr. Nermin Topaloğlu Avşar** ……..
İzmir Kâtip Çelebi University

Committee Members:

Assoc. Prof. Dr. Ozan Karaman ……..
İzmir Kâtip Çelebi University

Assist. Prof. Dr. Engin Baysoy ……..
İzmir Demokrasi University

Date of Defense: August 23, 2021

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Possible Biostimulative Effect of Low-Dose Photodynamic Therapy on HUVEC Cells

Abstract

The mechanism of Photobiomodulation (PBM) and Photodynamic Therapy (PDT) mainly depends on the production of intracellular reactive oxygen species (ROS). PBM is used for wound healing and cell proliferation while PDT is used to destroy pathogen and cancer cells. Low-dose PDT has also been shown to trigger pathogen and cancer cell proliferation, as well as the proliferation and differentiation of cells such as osteoblasts. However, the possible effects of low-dose PDT on endothelial cells inducing the differentiation and proliferation profile have not been investigated yet. This study aimed to enable the formation of low-dose ROS by using 808-nm wavelength laser radiation in the presence of Indocyanine green (ICG), and thus, to trigger various cellular mechanisms as in the PBM mechanism, for the differentiation of human umbilical vein endothelial cells (HUVEC). Since ROS production, nitric oxide (NO) release, and mitochondrial membrane potential (MMP) changes play an important role in PBM, these were analyzed. HUVECs were immunofluorescence stained with VEGF, PECAM-1, and vWf due to their importance in affecting the differentiation. As a result of the applications, it has been observed that low-dose PDT increased the cell viability by 20% and supported an approximately 20% increase in endothelial tubular structure formation compared to the control group. In PBM after triple light treatment, cell proliferation increased only by 7% and endothelial tubular structure almost 10%. Intracellular ROS and NO formed after the light applications play a key role for these outcomes to happen. It is assumed that ROS formed by low-dose PDT can trigger vasculogenesis and it was observed, that the biostimulative effect with low-dose PDT resulted in a higher increase in cell proliferation and differentiation compared to PBM.

Keywords: Photodynamic therapy; Photobiomodulation; 808-nm; Indocyanine Green; HUVEC cells.

Düşük Doz Fotodinamik Tedavinin HUVEC Hücreleri Üzerindeki Olası Biyostimulatif Etkisi

Öz

Fotobiyomodülasyon (PBM) ve Fotodinamik Terapi (FDT) mekanizması esas olarak hücre içi reaktif oksijen türlerinin (ROS) üretimine bağlıdır. PBM, yara iyileşmesi ve hücre çoğalması için kullanılırken, PDT patojen ve kanser hücrelerini yok etmek için kullanılır. Düşük doz PDT'nin ayrıca patojen ve kanser hücresi çoğalmasını ve ayrıca osteoblastlar gibi hücrelerin çoğalmasını ve farklılaşmasını tetiklediği gösterilmiştir. Ancak düşük doz PDT'nin endotel hücreleri üzerindeki olası farklılaşma ve çoğalma profilini indükleyen etkileri henüz araştırılmamıştır. Bu çalışmanın amacı, Indosiyanin yeşili varlığında 808 nm dalga boyundaki lazer ışıması kullanılarak düşük doz reaktif oksijen türlerinin oluşumunu sağlamak ve böylece insan umbilikal ven endotel hücrelerinin (HUVEC) farklılaşması için PBM mekanizmasında olduğu gibi çeşitli hücresel mekanizmaları tetiklemektir. Hücrelerde, hücre içi ROS üretimi, nitrik oksit (NO) salınımı ve mitokondriyal membran potansiyel (MMP) değişimi PBM'de önemli bir rol oynadığından, bu çalışmada bunlar analiz edilmiştir. HUVEC'lerin farklılaşmasını etkileyen önemlerinden dolayı hücreler VEGF, PECAM-1 ve vWf ile immünofloresan boyanmıştır. Uygulamalar sonucunda düşük doz PDT'nin kontrol grubuna göre hücre çoğalmasını %20 arttırdığı ve endotel tübüler yapı oluşumunda yaklaşık %20 artışı desteklediği görülmüştür. Üçlü ışık tedavisinden sonra PBM'de hücre çoğalması sadece %7 ve endotel tübüler yapı oluşumu ise %10 artmıştır. Işık uygulamaları sonrasında oluşan hücre içi ROS ve NO, bu sonuçların gerçekleşmesinde kilit rol oynamaktadır. Bu sonuçlar doğrultusunda, düşük doz PDT ile oluşturulan hücre içi ROS'un vaskülojenezi tetikleyebileceği varsayılmış ve düşük doz PDT ile biyostimülatif etkinin PBM'ye göre hücre çoğalmasında ve farklılaşmasında daha fazla artışa neden olduğu gözlemlenmiştir.

Anahtar Kelimeler: Fotodinamik Terapi; Fotobiyomodulasyon; 808-nm; İndosiyanin Yeşil; HUVEC hücreleri.

In memory of my beloved grandpa Mehmet Özkara.

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List of Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
COX	Cytochrome C oxidase
DCF	Dichlorofluorescin
DCFH-DA	2', 7'- dichlorofluorescin Diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EGM	Endothelial cell growth medium
FBS	Fetal bovine serum
HpD	Hematoporphyrin Derivate
HUVEC	Human umbilical vein endothelial cells
ICG	Indocyanine green
LED	light emitting diode
LLLT	Low-level light therapy
MMP	Mitochondrial membrane potential
MTT	2, 5-diphenyl tetrazolium bromide
NIR	Near-Infrared
NO	Nitric oxide
OLEDs	Organic light emitting diodes
PBM	Photobiomodulation
PBS	Phosphate buffered saline
PDT	Photodynamic therapy

PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PS	Photosensitizers
ROS	Reactive oxygen species
VEGF	Vascular Endothelial Growth Factor
VIS	Visible light
vWf	von Willebrand factor

List of Symbols

S^0	Singlet State
S^1	Excited Singlet State
T^1	Excited Triplet State
°C	Celsius

Chapter 1

Introduction

Tumors, wounds, or other health injuries are a part of life and are everywhere to find. These kinds of diseases have high priority in developing strategies to combat them. Light is one of the most important sources for healing or accelerating the requirements of healing. In healthcare, LEDs and Lasers are the most commonly used light sources. The selection of the right light source depends on the desired treatment effect since light can increase the cell viability or the opposite, it can kill cells. The killing mechanism is desirable in cases of pathogens or tumor cells, whereas the positive effect of increasing the cell viability, proliferation, and differentiation is desired for treatments such as wound healing. Different factors can affect the treatments and different effects of the used light sources and therapies.

To understand those differences, Photobiomodulation and photodynamic therapy mechanisms will be explained in the following.

1.1 Photobiomodulation/Low-Level Light Therapy

Photobiomodulation (PBM) or Low-level light therapy (LLLT) is defined as the use of non-ionizing photonic energy to trigger photochemical changes in cellular structures sensitive to photons. Low-power lasers or light-emitting diodes (LEDs) at visible (VIS, 400-700 nm) and near-infrared (NIR, 700-1000 nm) wavelengths are used in this treatment mechanism [1]. PBM has a photochemical interaction mechanism and is not thermal [2], which means the light is absorbed and causes biochemical reactions [3].

The light parameters are delivered at a low level which explains its terminology low level.

The first demonstration of `laser biostimulation` was in 1967, when the actually planned experiment by Dr. Endre Mester, at the Semmelweis Medical University went wrong and he accidentally came to the conclusion that laser had a biostimulative effect. He shaved the dorsal hair of mice, separated the subjects into two groups, and treated one of them with a low-powered ruby laser (694-nm). They did not develop cancer, and to his amazement, the treated group's hair grew back faster than the untreated group's [4].

Nowadays, PBM is an important part of healthcare. It has a wide range of uses, from pain management to supporting the recovery of tendinopathies, nerve injuries, osteoarthritis, and wound healing [2], thanks to its non-invasive nature.

Photons must be absorbed by electronic absorption bands belonging to a molecular chromophore [5] or photoacceptor for low-power visible light to have any effect on a living biological system, according to the first law of photobiology. A chromophore is defined as a molecule that gives the substance in which it is present a distinct color. Conjugated pi-electron systems and metal complexes are the two most common types of chromophores [6].

Another important thing to consider is the tissue's optical properties. In tissue, light absorption and scattering are wavelength-dependent. Both are far more abundant in the blue section of the spectrum than in the red, and hemoglobin, the major tissue chromophore has large absorption bands at wavelengths less than 600 nm [3]. For these reasons, the tissue has a so-called "optical window" that covers the red and near-infrared wavelengths (600-950-nm) and maximizes light penetration into the tissue shown in Figure 1.1

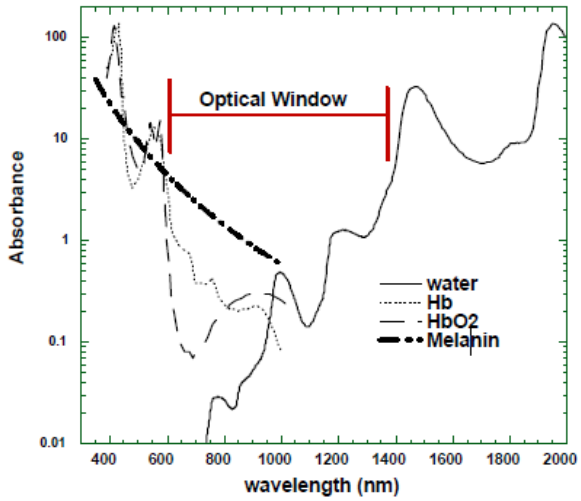

Figure 1.1: Optical window due to reduced absorption of red and near-infra-red in tissue [3]

1.1.1 Light Sources

PBM can currently be done with a wide range of light sources, including LEDs, organic LEDs (OLEDs), and lasers [8]. The effectiveness of PBM is based on the main parameters such as wavelength and dose. The optimal parameters are usually 1-1000 mW/cm^2 and for power density and energy density the parameters ranging from 0.1-100 J/cm^2. The time is measured in seconds, minutes, or hours. A photo acceptor molecule in the cell or organism must be able to absorb the wavelength to induce a desirable biostimulative effect.

Laser light has the advantage of being able to be amplified by stimulated emission and can be used for the treatment of different types of diseases [9]. The properties of laser light are monochromacy, coherence, and collimation. Laser light is used in a variety of sectors due to its unique properties. One of the fields that benefit from light energy is medicine. Lasers are utilized for a variety of applications ranging from diagnosis and therapy of different kinds of diseases.

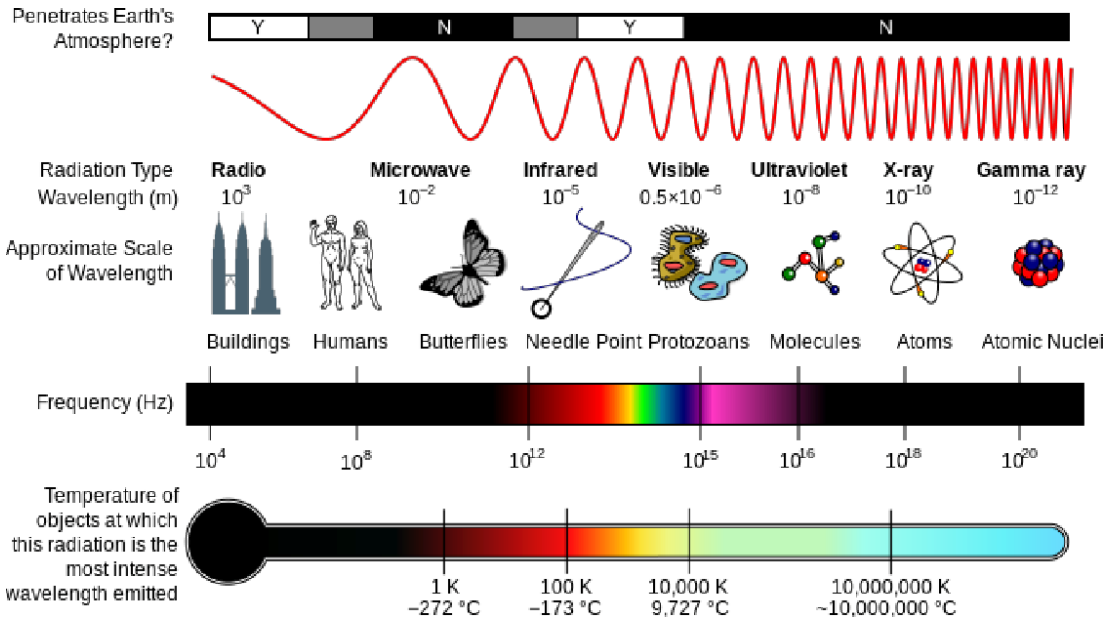

Figure 1.1.1: The electromagnetic spectrum [7]

A photoacceptor molecule in the cell or organism must be able to absorb the wavelength of the light which is emitted from a light source. When light enters tissue, it is either scattered or absorbed, and the amount of both processes varies depending on tissue type and light wavelength [10]. Multiple scattering in a turbid medium causes a light beam to spread out and lose its directionality. As shown in Figure 1.1, chromophores within the tissue, such as hemoglobin, myoglobin, and cytochromes play a big role in absorption.

The radiation used in PBM and Photodynamic therapy (PDT) is the light of the VIS and NIR regions shown in Figure 1.1.1. The wavelength necessary to accomplish a specific therapeutic effect is determined by the chromophore within the tissue, as well as the PS employed and the tissue's optical characteristics in terms of PDT [11].

The connection and importance between different parameters and their effects on tissue or different cells are shown in a study, where two different doses are used. They used in their study 655-nm and 808-nm laser devices. It can be obtained, that different types of cells react differently to those light irradiations. 808-nm irradiation at an energy density of 1 J/cm^2 promoted wound healing on keratinocyte cells, whereas in fibroblast cells no relevant changes were obtained with the same wavelength. 655-nm was more effective on fibroblast cells in terms of wound healing. However, 808-nm irradiation with an energy density of 3 J/cm^2 was surprisingly promoted fibroblast cell

viability [12]. This example shows the importance of the right parameters to achieve the best possible and desired solutions during an application.

1.1.2 Mechanism of PBM

PBM is the use of non-ionizing radiation or light to stimulate numerous biological functions, especially in the visible and near-infrared regions of the electromagnetic spectrum. It involves administering low-level visible or near-infrared light to a target tissue or cells without causing any harm in form of heat energy by stimulating multiple signaling pathways in the cell metabolism to induce various biochemical responses [13]. Depending on the type of target tissue and cell, these responses may result in cellular and tissue effects such as adenosine triphosphate (ATP) production, cell proliferation, wound healing, DNA synthesis, increased blood flow, inflammation, or oxidative stress [14].

Cytochrome C is an important part of the electron transport chain, which is responsible for cellular metabolism [15]. Light absorption by Cytochrome C oxidase (COX) stimulates the electron transport chain, increasing the generation of ATP within the mitochondria, followed by the production of reactive oxygen species (ROS), an increase in mitochondrial membrane potential (MMP), and a protein gradient across the cell and mitochondrial membrane [16]. PBM increases MMP, resulting in enhanced electron transport. Increased MMP is thought to cause an increase in ROS in the past.

In the long-term follow-up of cell activities, ATP synthesis, intracellular ROS generation, and nitric oxide (NO) release indicates their impacts. As a result, the fundamental action of light on cells via PBM is a shift in mitochondrial membrane potential following light absorption by COX [13].

When tissue is damaged, the cell's ability to produce ATP is compromised, slowing the cell's metabolism as a protective measure. PBM aids in the re-establishment of the oxidative process, which in turn aids in the restoration of normal cellular activity [17].

In addition to ATP, laser stimulation produces free NO and induces ROS generations. NO is a strong vasodilator as well as a critical cellular signaling molecule involved in

several physiological functions [18]. ROS has been shown to influence many important physiological signaling pathways, including the inflammatory response. Increased NO and improved ROS levels combine to generate a favorable environment for faster signaling, resulting in a reduction in inflammation [19].

The presence of NO as a signaling agent is required for the activation of various cellular pathways [20]. COX is inhibited by this substance, which is produced in the mitochondria. The separation of NO from COX increases respiratory rate [21]. In both isolated mitochondria and entire cells, light can reverse the inhibition produced by NO binding to COX [22]. Increased COX activity could explain the greater ATP levels, which are one of the most common alterations documented *in vitro* following PBM. PBM can also protect cells from cell death caused by NO.

1.2 A Different Strategy to Induce Low-Level Light Therapy

PDT is another therapeutic procedure in which light is widely employed as a tool. The basic goal of PDT is to kill cancerous and non-cancerous cells by using light-sensitive compounds, called photosensitizers (PS) that absorb a specific wavelength and initiate a chain of energy transfer reactions that results in the creation of ROS [23]. The degree of phototoxicity on the cells is determined by the amount of ROS created when the PS absorbs light. As a general rule in PDT, the photosensitizer and light have no adverse effect on the target when used alone. When they are used combined, a large amount of ROS is produced as hazardous chemicals, which kill the target cells. The amount of quantum oxygen yield of the photosensitizers after irradiation determines their toxicity. Low levels of ROS serve a crucial function in cellular life cycles like proliferation and homeostasis [16]. More crucially, the production of ROS has long been recognized as an important process in disease resistance, cell-mediated immunity, and microbiocidal activity, all of which help to protect our bodies against invading organisms [24]. An imbalance between ROS generation and detoxification could produce oxidative stress in cells with high levels of ROS. This would cause oxidative damage to cellular constituents (such as proteins and lipids), apoptosis or necrosis, and, most likely, the encouragement of cancer-causing mutations [25]. The presence

of ROS can also signal a variety of cellular activities that fluctuate in type and over time. This fact was also the starting point for PBM. PDT used in low doses promoted endothelial cell proliferation and VEGF expression in nude mice brains [26]. Another study by Jayasree et al. showed that two different lasers, He-Ne and Nd: YAG laser, had also a positive effect on the wound healing of rats [27]. It resulted in an accelerated wound healing process in groups with low-dose PDT compared to the control groups. These studies show that laser parameters have a key factor and have effects comparable to those of PBM. PDT deviates from the killing mechanism in low doses and is used to accelerate cell proliferation.

1.2.1 Mechanism of PDT

PDT uses the combination of photosensitizing drugs, called PS, and light [28]. PDT is a non-invasive treatment for non-oncological conditions as well as malignancies of various forms and locales. The photosensitizer molecules absorb light of the correct wavelength, causing activation processes that cause undesirable cells to be selectively eliminated. It is also used to treat chronic inflammation, and it is a promising new therapeutic option for drug-resistant bacterial infections. The molecular mechanism of PDT has three main components; the photosensitizer, light with appropriate wavelength, and molecular oxygens [29,30].

The photodynamic response has two major processes. Both are reliant on oxygen molecules found inside cells. Both systems have a similar first stage. After entering the cell, a photosensitizer is exposed to light with a wavelength that corresponds to the PS absorption spectrum and is transformed from the singlet energy state S° to the excited singlet state S_1 due to photon absorption. The remaining energy leads a photosensitizer molecule to the excited triplet state T_1 - the appropriate, therapeutic form of the compound - while a portion of the energy is radiated as a quantum of fluorescence. In other words, in the low-energy molecular orbital of PS, there are two electrons with opposite spins in the ground state. One of these electrons gets excited to a higher energy molecular orbital when light is absorbed, but it does not change its spin. PS cannot engage in reactions with cellular substrates in its singlet excited state due to its short lifespan, which ranges from nano to picoseconds [31,32].

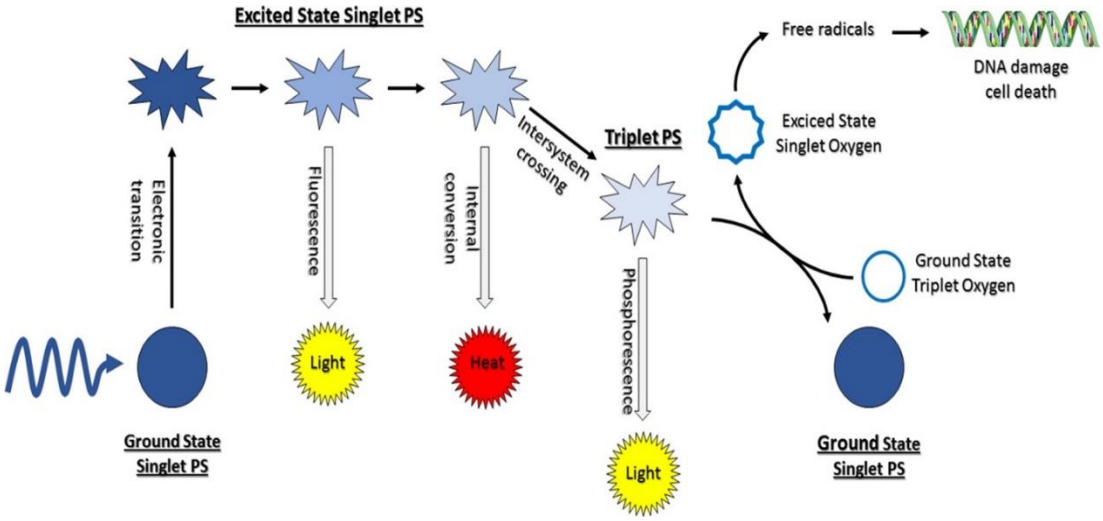

Figure 1.2.1: Modified Jablonski diagram [33]

1.2.2 Photosensitizers

Apart from light and molecular oxygens, PSs are one of the three essential components of PDT. These dyes are compounds that may absorb light of a given wavelength and cause photochemical or photophysical processes [34]. An ideal PS should have different properties and characteristics. Some of those characteristics are listed down belove:

- Chemical purity
- Selectivity for specific cells
- Chemical and physical stability
- High singlet oxygen yield
- Photosensitivity only when exposed to a specific wavelength
- Minimal cytotoxicity in absence of light
- Low-cost [28,33,34,35].

PS agents are light-transmitting natural or synthetic structures [36]. Porphyrin derivatives, chlorins, phthalocyanines, and porphycenes are the four primary types of photosensitizers, and they all have various photochemical and photophysical features in terms of methods of action and light activation [37]. The PSs can also be categorized

into different generations. The first-generation PS`s are porphyrin derivates and include hematoporphyrin and its derivates called Hematoporphyrin Derivate (HpD) [38].

The clinical use of first-generation PSs was limited due to their drawbacks such as their poor chemical purity or its activation wavelength which is too short to achieve a good tissue penetration. These drawbacks led to improve more efficient PSs. The second-generation PSs are based on porphyrin structures or are chlorine-based structures such as chlorin e6. Other second-generation PSs, such as the new mitochondria-targeting photosensitizers, was created to fulfill specific needs. The composition and structure of the second-generation PSs are clear when compared to the first-generation photosensitizer, and the photosensitivity, absorption spectrum, and tissue selectivity have all been considerably enhanced. Third-generation PSs are made up of first- and second-generation PS that has been coupled to various modifiers such as biologicals, antibodies, and nanoparticles [39]. They are based on synthetic substances which can focus on destroying tumor tissues and minimize the damage on the healthy tissue [33].

1.2.3 The Importance of the Dose in PDT

The basic goal of PDT is to kill cancerous and non-cancerous cells by using light-sensitive compounds (photosensitizers) that absorb a specific wavelength and initiate a chain of energy transfer reactions that results in the creation of ROS [23]. The degree of phototoxicity on the cells is determined by the amount of ROS created when the photosensitizer absorbs light. Generally, in PDT, the photosensitizer and light have no adverse effect on the target when used alone. When they are used combined, a large amount of ROS is produced as hazardous chemicals, which kill the target cells [40].

The radiation used in PDT is light of the visible and near-infrared regions. The wavelength required to accomplish a specific therapeutic effect is determined by the PS employed as well as the tissue's optical characteristics [41]. Lasers are suitable to light sources for PDT due to their ability to deliver intense light with a high degree of coherent monochromaticity. This property enables the light beam to be focused into an optical cable with little scattering energy loss. The light source for PDT must have

appropriate spectral qualities that match the maximal absorption wavelength range of the PS utilized to generate enough ROS to have a cytotoxic effect [42].

In Photofrin-mediated PDT, for example, the required light dose is normally 50–500 mW but when it comes to second-generation sensitizers, which have stronger light absorption, this dose changes [11]. These studies are an indication of the importance of the parameters used during PDT, such as wavelength, optical power, and energy density.

ROS has dose-dependent effects on cell functioning. ROS can be harmful to cells and cause physiological dysfunction at high levels, but at low levels, they are vital for cell signaling because they can change redox-sensitive proteins involved in cell proliferation and differentiation [16, 43]. Low-energy laser irradiation produces a little amount of ROS, but high-energy laser irradiation produces a considerable amount of ROS, potentially exposing cells to chronic oxidative stress. Cell growth is slowed as a result of oxidative damage.

A study was done by Bölükbasi Ates et al. with indocyanine green (ICG) and an 809-nm wavelength laser resulted in the enhanced cellular activity of osteoblast cells [44]. The decisive factor here was the selection of the laser parameters with 10 W output power, 50 mW/cm^2 power density, and 0.5, 1, and 2 J/cm^2 energy densities with an irradiation time of 10, 20, and 40 s, respectively. Another study was done on human fibroblast cells resulted in accelerated closure of scratch wounds [45]. They used a 660 nm wavelength with a power density of 30 mW/cm^2 for 30 seconds and output power of 1 J/cm2 in combination with 5-ALA as a PS. These studies prove that with low parameters, PDT has a biostimulative effect. And thus, it differs from the traditional use of the PBM. These studies are an indication of the importance of the parameters used during PDT, such as wavelength, optical power, and energy density [46].

1.2.4 Biomedical Applications of the Different Low-Level Light Therapies

PBM's therapeutic benefits are influenced by several factors, including wavelength, energy density, and application time [47]. PBM has many different treatment purposes such as wound healing, cell viability, and pain relief, and more [48]. PBM promotes

cell proliferation, migration, and remodeling by regulating the expression of genes involved in cell proliferation, migration, and remodeling. Using human fibroblast cells, an in vitro study done by Ayuk et al. found that PBM aids in promoting the remodeling phase of wound healing by lowering matrix breakdown and enhancing matrix synthesis [49]. Irradiation of fibroblast with 660 nm wavelength resulted in stimulation of their differentiation potential. Another study done by Mokoena et al. has shown, that a low-power He-Ne laser increased the growth factor of VEGF in murine myocardium, which is necessary for wound healing [50]. PBM is also used by physical therapists, in dermatology, dentists, and other sectors of health care for different purposes [51].

PDT is a popular method in cancer treatment. It is used for the treatment of various types of cancers [52], such as breast [53], lung or skin cancer [54]. The milestone for cancer treatment of PDT dates back to the publication of the effect of HpD with light by a few patients with bladder cancer in the late 1970s [55]. Besides cancer treatment, PDT is used for destroying microorganisms, such as acne vulgaris. An in vitro study done by Soria-Lozano et al has shown PDT was effective in killing *S.mutans* and *S.anguis strains* [56].

Newer studies have shown, that low-dose PDT had positive effects on wound healing, increasing cell proliferation and differentiation. The examples of wound healing [48, 49] in different cell types confirm this assumption, that low-dose PDT has an opposite effect compared to high-dose PDT. Low-dose PDT effects are comparable to those achieved with PBM.

Since wound healing plays an important role in biomedical researches, endothelial cells provide a good basis for studying the effects of low-dose PDT in more detail. A study done on HUVECs, which are endothelial cells, has shown, that PBM with 660 and 780 nm light irradiation had different effects on its cell viability [57]. While red laser enhanced the cell viability and total concentration of proteins in those cells, IR laser had the opposite effect on cell viability by maintaining the total protein concentration compared to the red light irradiated groups.

The purpose of this study is to compare the effect of PDT at low dose with the combination of ICG as a PS, also used in low dose, with PBM on HUVECs. Since

low-dose PDT has shown PBM effects on different studies. NIR light has a deep penetration capacity into tissue due to this characteristic, it has wide biomedical usage, such as wound healing. The endothelial cells are important for the vascularization and angiogenesis during a wound healing process due to this 808-nm, NIR light source has the ability of deep penetration. Therefore, NIR was used for this study to have more effective biostimulation. ICG is best excited at around 800 nm and works well used with 808 nm laser irradiation as PS. The analyzes to determine the biostimulative effect was primarily based on cell proliferation and cell differentiation and were done in form of MTT assay and tubular structure formation. ROS, NO, and also MMP are factors affecting the biostimulative effect were analyzed. Since vascularization plays an important role in terms of diseases related to endothelial cells, protein production is another indication for vascularization and cell differentiation. To obtain this, immunofluorescence staining was performed.

Chapter 2

Materials and Methods

2.1 Light Source and Optical Setup

In this study, an 808-nm diode laser (Teknofil, Istanbul) was used as a light source with a maximum output power of 3 Watts. The parameters used for this study are given in Table 2.1. The optical fiber of the laser device was positioned vertically and the fiber tip was 10 cm distant from the cell culture plates on an optical table as shown in figure 2.1.

Table 2.1: Light Parameters

Wavelength (nm)	Output Power (mW)	Energy Density (J/cm^2)	Irradiation Time (s)
808 nm	600 mW	1 J/cm^2	5 s

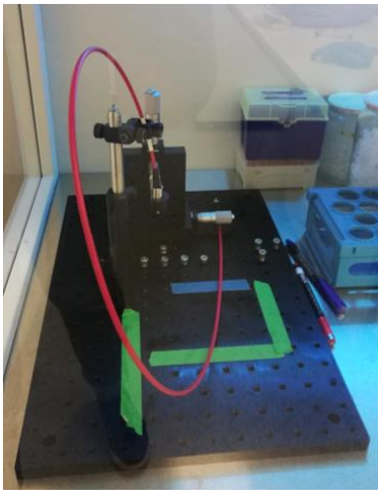

Figure 2.1: Optical setup that transmitted light to the cells in the wells of a plate.

2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Ege University Research Group of Animal Cell Culture and Tissue Engineering Laboratory and cultured in a humidified atmosphere with 5% CO_2 at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) from Sigma-Aldrich (St. Louis, MO, USA) containing 5% fetal bovine serum (FBS) from Gibco (Dublin, Ireland), 1% L-glutamine from Sigma-Aldrich (St. Louis, MO, USA) and 1% antibiotic (penicillin). On a 96-well plate, three wells with identical light intensity were identified, and HUVEC cells were plated in each well and cultured in a cell culture medium for 24 hours at 37 °C.

2.3 Photosensitizer

ICG is a dark green, cyanine dye with anionic characteristics and is a fluorescent agent widely used in biomedical fields. In the wavelength range of 600 to 900 nm, excited by approximately 800 nm wavelength of light. ICG has the appealing properties of low toxicity and strong absorptance. The molecular formula of ICG is $C_{43}H_{47}N_2NaO_6S_2$ and the molecular weight is 774.96 g/mol.

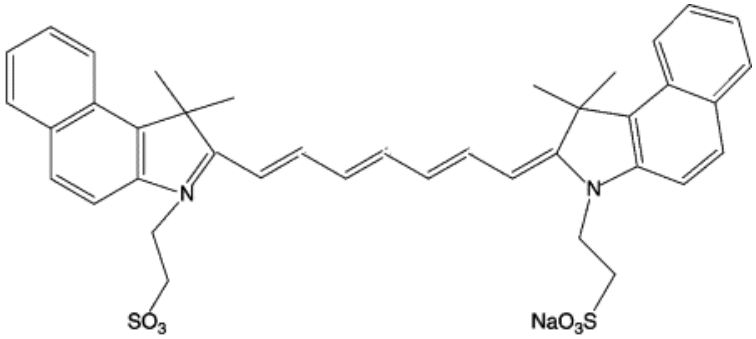

Figure 2.3: Chemical structure of ICG [58].

For this study, 1.93 mg of ICG purchased from Santa Cruz (Dallas, Texas, USA) was dissolved in 500 µL DMEM. The obtained concentration of 5 mM was then diluted with DMEM to the concentration of 0.05 µM, 0.1 µM, 0.25 µM, 0.5 µM, 1 µM, 2.5 µM, and 5 µM.

2.4 Cytotoxicity of ICG on HUVECs

Seven different concentrations of ICG (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, and 5 µM) were prepared in serum-free medium (DMEM) in the dark and applied on HUVEC cells to observe the possible toxic effect of the photosensitizer on the cells and finally to determine the ideal concentration for the low-dose PDT applications. ICG solutions with different concentrations were added onto the cells and were incubated for 1h. After the incubation, the cells were washed once with phosphate buffered saline (PBS) from Sigma-Aldrich (St. Louis, MO, USA) and endothelial cell growth medium (EGM) purchased from Lonza (Basel, Switzerland) was added. After 24h, cell viability analysis was performed by 2, 5-diphenyl tetrazolium bromide (MTT) assay purchased from Sigma-Aldrich (St. Louis, MO, USA) measuring the absorbance with a microplate reader at 570 nm (Multimode Microplate Reader Biotek Synergy HTX, Biotek, Winooski, VT, USA).

2.5 Experimental Procedures of PBM and Low-Dose PDT

In this study, two groups were formed for both PBM and low-dose PDT applications; Single and Triple treatment groups and each of them consists of Control and 1 J/cm^2 energy density groups. The cells in the single treatment groups were irradiated only once throughout the experiments. The cells in the triple treatment groups were received daily light irradiation with a time interval of 24 hours. First of all, the seeded cells were incubated for 24 h for cell attachment on the surface of the 96-well plates. Then for low-dose PDT groups, ICG was added and incubated for an hour. Afterward, the cells were washed with PBS twice and EGM was added. For the PBM applications, only EGM was added and incubated for 1 h. Before laser application, the optical table and the laser device were sterilized for 30 min under UV before irradiating cells. After the incubations, the cells were irradiated by an 808-nm diode laser at 1 J/cm^2 energy density. To perform a triple treatment, this application was repeated three times at 24-hour intervals. The medium of HUVEC cells was changed every day with EGM.

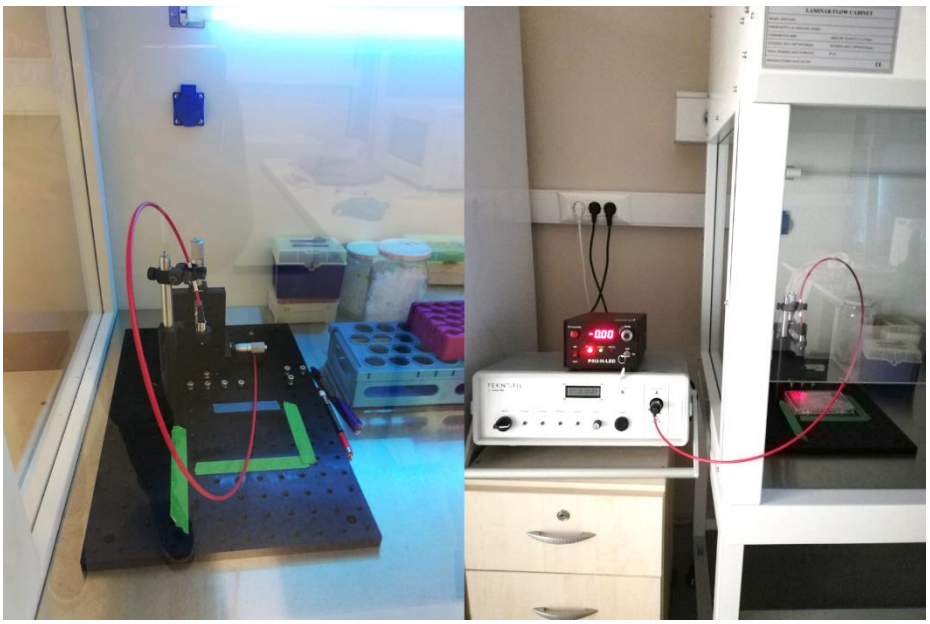

Figure 2.5: Optical setup under UV and during light applications.

2.6 Microscopic Analysis of HUVECs

On day 1, day 2, day 3, day 4, and day 7, HUVECs were evaluated throughout the experimental procedure. An inverted microscope was used to capture microscopic images of living cells (Olympus, CKX41). Each of the three wells had 10 images (20X magnification) taken for morphological study of the cells. The total length of the tubular structures was measured with the aid of the "Angiogenesis Analyzer" plugin for ImageJ software (NIH, Bethesda, MD, USA) and calculated relative to the control group as a percentage.

2.7 Cell Viability Analysis

MTT assay was used to calculate the viability of HUVEC cells. First, the stock 5 mg/ml MTT solution prepared, then added to the wells to be 10% of MTT solution and 90% of DMEM without serum. After 2h of incubation, MTT solution was removed and Dimethyl Sulfoxide (DMSO) solution purchased from Merck (Darmstadt, Germany) was added with the same volume of 100 µL. The microplate reader measured absorbance values at 570 nm after 30 minutes of DMSO incubation. The MTT experiment was completed in complete darkness.

2.8 Analysis of Intracellular ROS Generation

The intracellular ROS assay was performed by using a non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) from Sigma-Aldrich (St. Louis, MO, USA). In the presence of ROS, DCFH-DA can be transformed into dichlorofluorescein (DCF), a fluorescent molecule. First, cells were seeded in 96-well plates and incubated in a humidified atmosphere with 5% CO_2 at 37°C for 24h. After 24h of incubation, the cells were washed once with PBS, then 0.01 mM of DCFH-DA was added, and incubated for 45 minutes. Afterward, they were washed twice with PBS and the particular applications were performed for both PBM and low-dose PDT groups. At the end of the applications, the fluorescence intensity of DCF was measured using the microplate reader directly after the applications with excitation wavelengths of 485/20 nm and an emission wavelength of 535/20 nm.

2.9 Analysis of NO Release

Griess reagent kit from Biotium (Fremont, CA, USA) was used to assess the amount of NO released as a result of laser treatments containing sulfanilic acid and N-(1-naphthyl)ethylenediamine. The breakdown product of NO, nitrite, reacts with sulfanilic acid and N-(1-naphthyl)ethylenediamine to generate a dye molecule that can be measured spectrophotometrically. The identical volume of Griess reagent and the supernatant solution from each sample were mixed and incubated for 30 minutes at room temperature, 24 hours after the light applications were completed. The absorbance values of each sample were measured using a microplate reader at a wavelength of 548 nm on day 1, day 2, and day 3 after light applications. The amount of nitrite in each sample was calculated using the standard curve's formula as an indicator of NO released after applications.

2.10 Analysis of MMP change

The JC-1-Mitochondrial Membrane Potential assay kit from Abcam (Cambridge, UK) was used to assess the change in MMP during light applications according to the manufacturer's instructions. The cells were rinsed with dilution buffer and then incubated with 10 M of JC-1 solution for 10 minutes at 37°C before light applications.

The cells were washed twice with the dilution buffer after incubation, and the appropriate application protocols were carried out in each PBM and PDT group. The fluorescent signals were read instantly at an excitation wavelength of 475 nm and the emission wavelengths of 595 nm and 535 nm with CLARIOstar Microplate Reader (BMG LABTECH, Germany), and the images of the experimental groups were captured with a fluorescent microscope (Olympus CKX41, Olympus Co. Ltd., Tokyo, Japan) with a magnification of 20X. CellSens Imaging Software was used to create these images where the red fluorescence represented the hyperpolarization of cells.

2.11 Immunofluorescence Staining

On day 7 after single and triple treatments were performed on both groups, PBM and PDT, HUVEC cells seeded in 96-well plates were washed twice in PBS before being fixed with 4% paraformaldehyde purchased from Sigma-Aldrich (St. Louis, MO, USA) at 4°C for 30 minutes for immunofluorescence staining. Afterward, samples were immersed with 0.1% Triton X-100 in PBS for 1 hour and blocked with 1.5% Bovine Serum Albumin (BSA) in PBS for 2 hours. The samples were then incubated overnight at 4°C with primary antibodies in PBS containing 1% BSA, as directed by the manufacturer. Primary antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) included VEGF (cat. no. sc-53462; 1:50), PECAM-1 (cat. no. sc-376764; 1: 50) and vWf (cat. no. sc-365712; 1: 50). Fluorescence secondary antibody included m-IgGκ BP-PE (cat. no. sc-516141; 1:50). Before usage, the secondary antibody was diluted with 1% BSA. Each sample was also stained with 4,6-diamidino- 2-phenylindole (DAPI) to image the cell nuclei. Using an inverted fluorescent microscope, pictures of the expression patterns of VEGF, PECAM-1, and vWF were captured with the same exposure time and light intensity. (Olympus CKX41, Tokyo, Japan).

2.12 Quantitative Real-Time PCR Analysis for the Expressions of vWf and PECAM-1 genes

On day 7 after all laser irradiations were completed, total cellular RNA using total RNA purification kit obtained from GeneAll Biotechnology, co., ltd. (Seoul, Korea) was isolated for each group. Afterward, the conversion of the extracted purified RNA to cDNA using HiScript III 1st Strand cDNA Synthesis Kit purchased from Vazyme Biotech Co., Ltd (Nanjing, China) was performed for the use in RT qPCR process. The gene specific primers (forward and reverse) used for RT qPCR including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as a housekeeping gene, PECAM-1 and vWf purchased from Oligomer (Ankara, Turkey) are shown in Table 2.12. StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, USA) was used for RT qPCR process. The last step of analyzing the expression of each gene for each group was carried out using the GAPDH expression with StepOne Software v2.3.

Table 2.12: Forward and reverse primers

Genes	Direction	Gene sequences
GAPDH	Forward	GAAATCCCATCACCATCTTCC
	Reverse	CCAGCATCGCCCCACTT
vWf	Forward	CCCATTTGCTGAGCCTTGT
	Reverse	GGATGACCACCGCCTTTG
PECAM-1	Forward	GCTGACCCTTCTGCTCTGTT
	Reverse	TGAGAGGTGGTGCTGACATC

2.13 Temperature Measurement During Light Application

Temperature difference, in form of too much heat, can cause unwanted side effects during the application. To control the temperature and exclude any side effects related to heating, the temperature was measured by a thermal camera (Testo, Thermal

imager). The measurement was taken during all light applications for both, PBM and also PDT groups. The temperature was measured at the beginning and the endpoint of the application due to the short period of 5 seconds. The results were taken from the subtraction of the end temperature and the start temperature.

2.14 Statistical Analysis

Within each experiment, at least three samples from the same group were used in each experiment and each experiment was repeated three times. All data was first evaluated using one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 9 software. The *p* values less than 0.05 were considered statistically significant.

Chapter 3

Results

3.1 Cytotoxicity of HUVECs

Figure 3.1 shows the cell viability after 24 hours of incubation with different ICG concentrations. The ICG concentrations were obtained by diluting them in DMEM. The potential cytotoxicity effect of ICG on HUVECs was determined by the MTT assay. It was observed that ICG alone in prescribed concentrations (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, and 5 µM) did not show any toxic effects. The concentrations tested are roughly comparable to the control group. There is a slight decrease observed in 0.1 µM, 0.5 µM, 1 µM, and 2.5 µM. With an increase of approximately 25 %, the concentration of 5 µM showed the highest cell viability compared to control. The concentration used for the study was 0.1 µM.

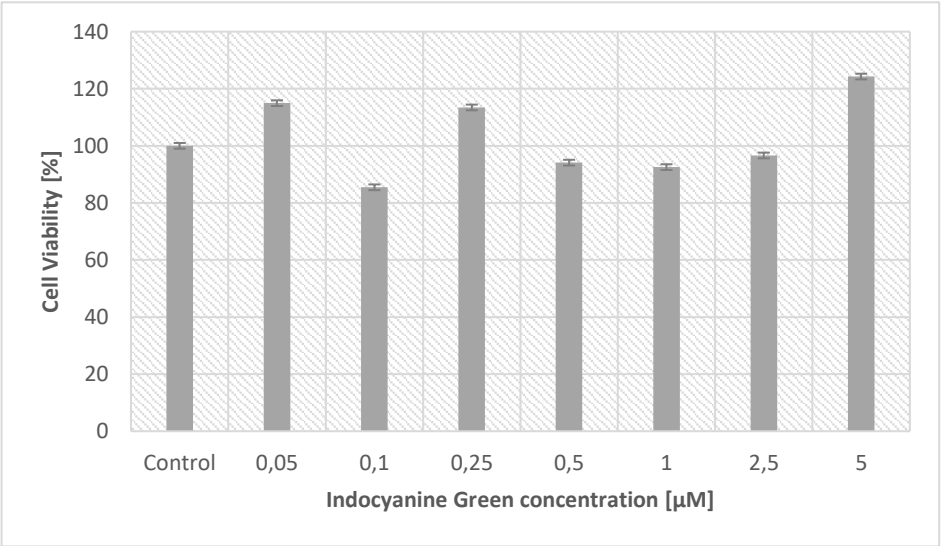

Figure 3.1: Cell Viability after 24 h incubation of HUVECs with different concentrations of ICG

3.2 Cell Viability of HUVECs after PBM and PDT applications

The measurement for the metabolic activity of HUVECs was carried out on the 7th day with the MTT assay. As shown in Figure 3.2a, there is an increase of approximately 3% after a single light treatment of PBM. The value increased to 7% after triple treatment of PBM. In general, it can be observed that compared to the control group in both treatments there is an increase. Figure 3.2b shows the cell viability in percentage after single and triple light treatment of PDT. While the increase after a single treatment of PDT is only 5%, this value increases to 20% after triple treatment of PDT. The comparison of PBM with PDT groups shows that the result of PDT resulted in clearly higher cell viability than PBM for both treatment groups. The difference between both single treatments is approximately only 2% whereby the difference after triple treatment is significantly higher with a difference of approximately 13%.

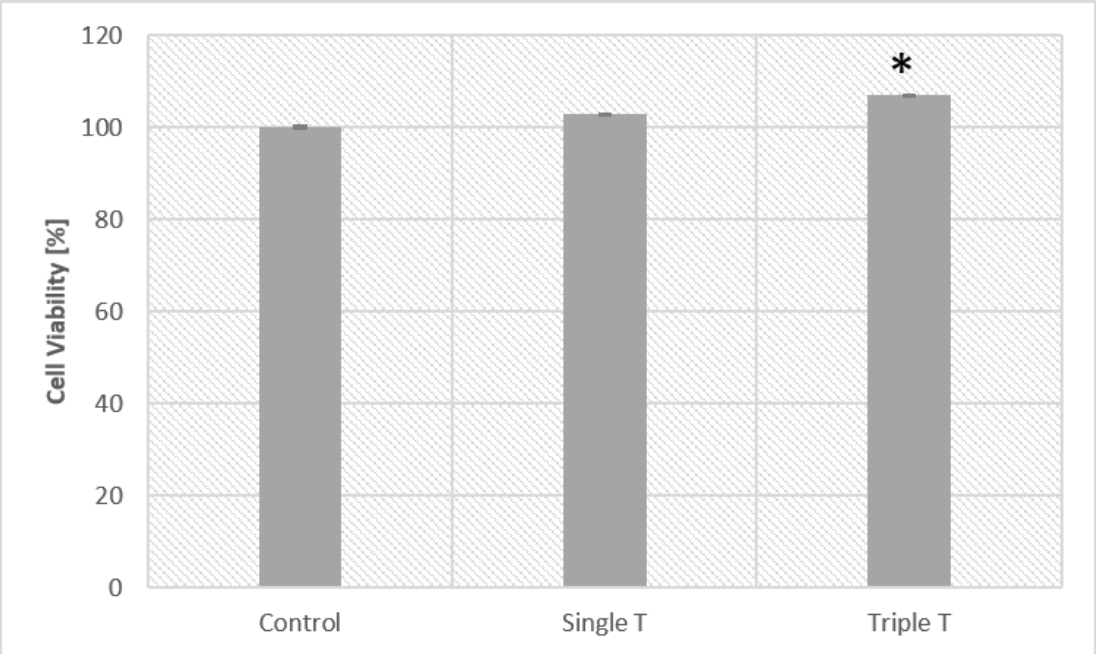

Figure 3.2 a: Cell viability of HUVECs after PBM (single and triple treatment; 1J/cm^2) on 7th day (p<0.05, where * symbolizes significant differences compared to the control group)

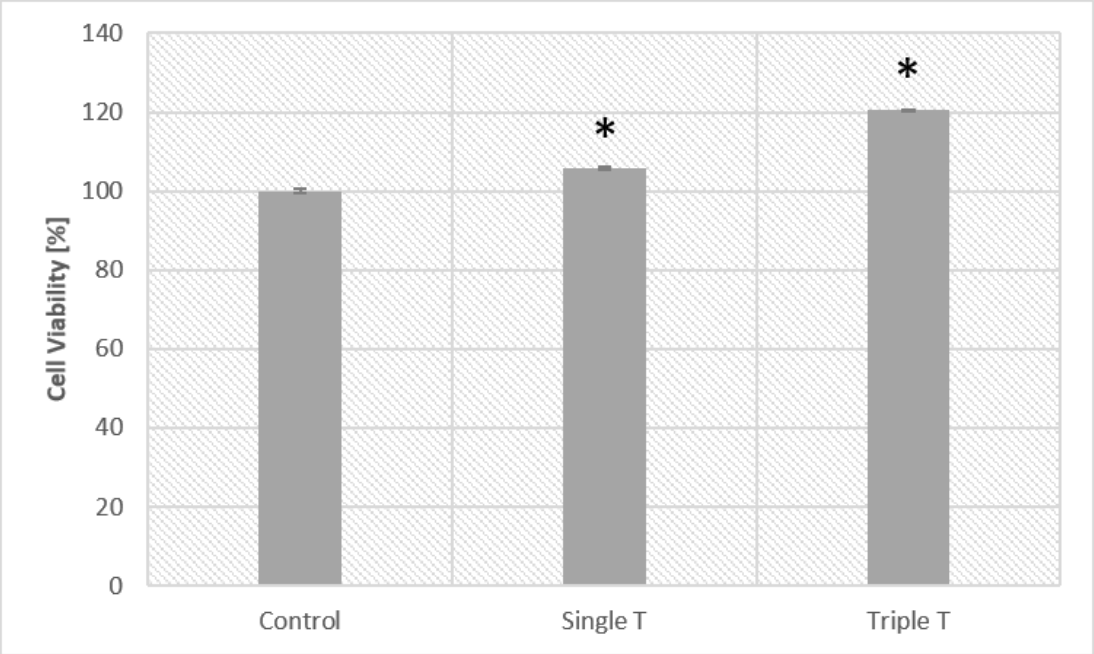

Figure 3.2 b: Cell viability of HUVECs after PDT (single and triple treatment; 1J/cm^2) on 7th day (p<0.05, where * symbolizes significant differences compared to the control group)

3.3 Microscopic Analysis of HUVECs after Light Treatments

Figure 3.3a and Figure 3.3b show images of the cells obtained by light microscopy and it was observed that HUVECs successfully proliferated and elongated in PBM and low-dose PDT groups, respectively. To examine whether PBM and low-dose PDT applications contribute to the formation of tube-like structures in vitro, we performed angiogenesis analysis by using phase-contrast images with the aid of the "Angiogenesis Analyzer" plugin for ImageJ software (Figure 3.3c and Figure 3.3d).

Compared to control, we observed after a single treatment, that the formation of endothelial cell tubular structure was increased to 111± 2% in PBM, whereas in group PDT the tube formation was approximately 8% below the control group value.

After triple treatment, compared to control, we observed that the formation of endothelial cell tubular structures increased to 118 ± 3%, 110 ± 2%, in the PDT group and PBM group, respectively.

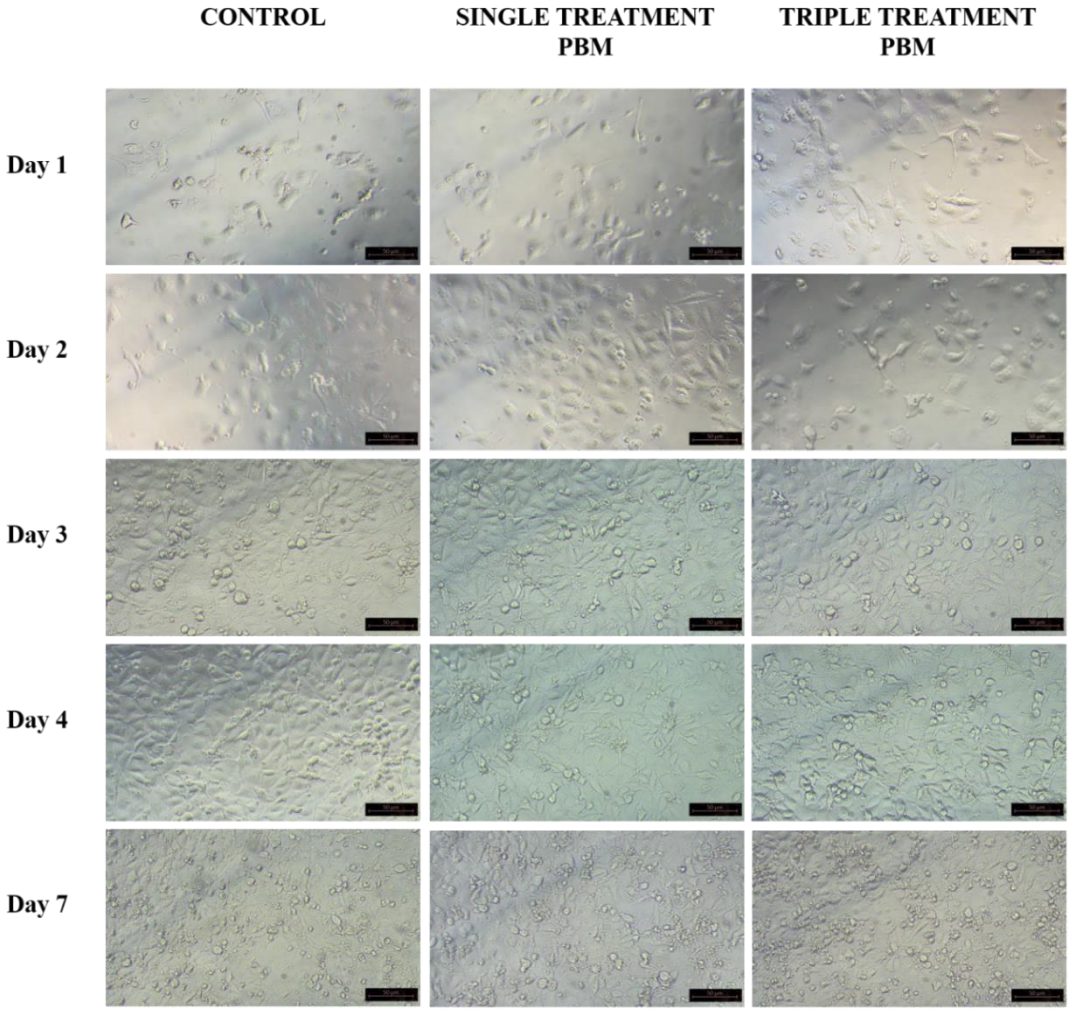

Figure 3.3a: Microscopic images of HUVECs with 20X magnification (scale bar: 50 µm) in control, PBM single treatment, and PBM triple treatment at days 1, 2, 3, 4, and 7.

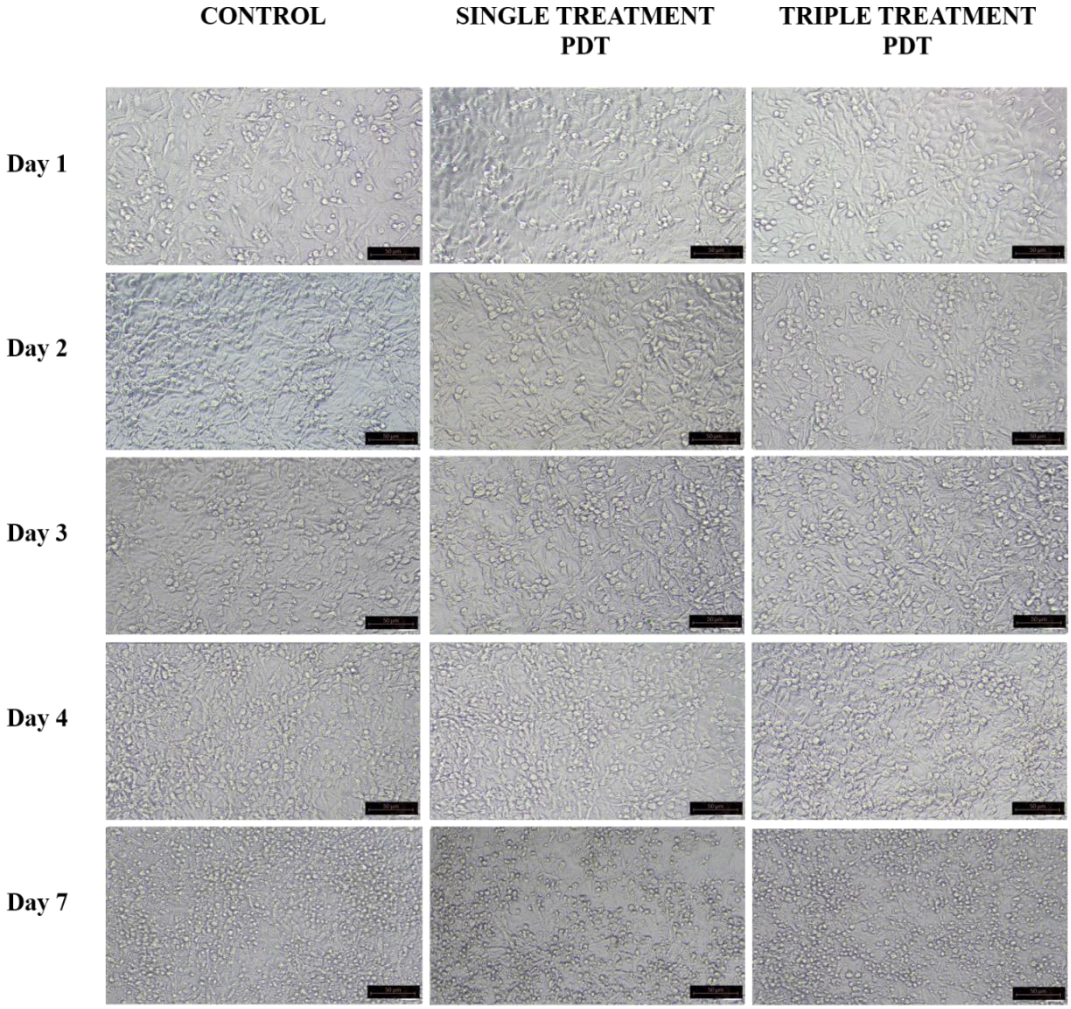

Figure 3.3b: Microscopic images of HUVECs with 20X magnification (scale bar: 50 µm) in control, PDT single treatment, and PDT triple treatment at day 1, 2, 3, 4, and 7.

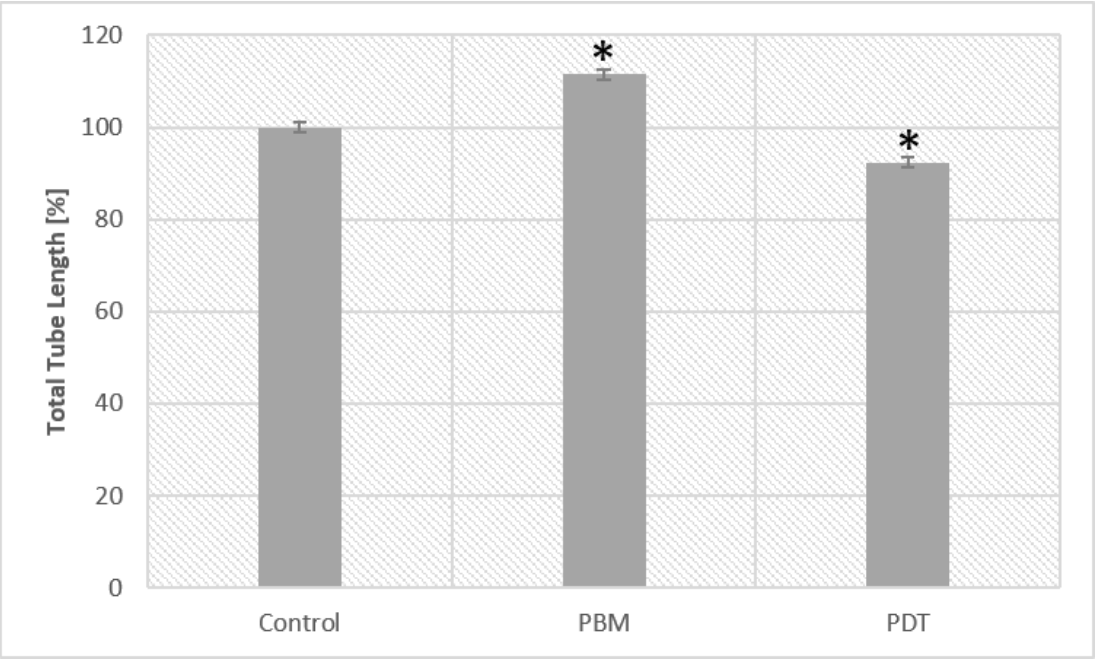

Figure 3.3c: The total length of the tubular structures in PBM and PDT after single treatment on day 7 (p<0.05, where * symbolizes significant differences compared to the control group)

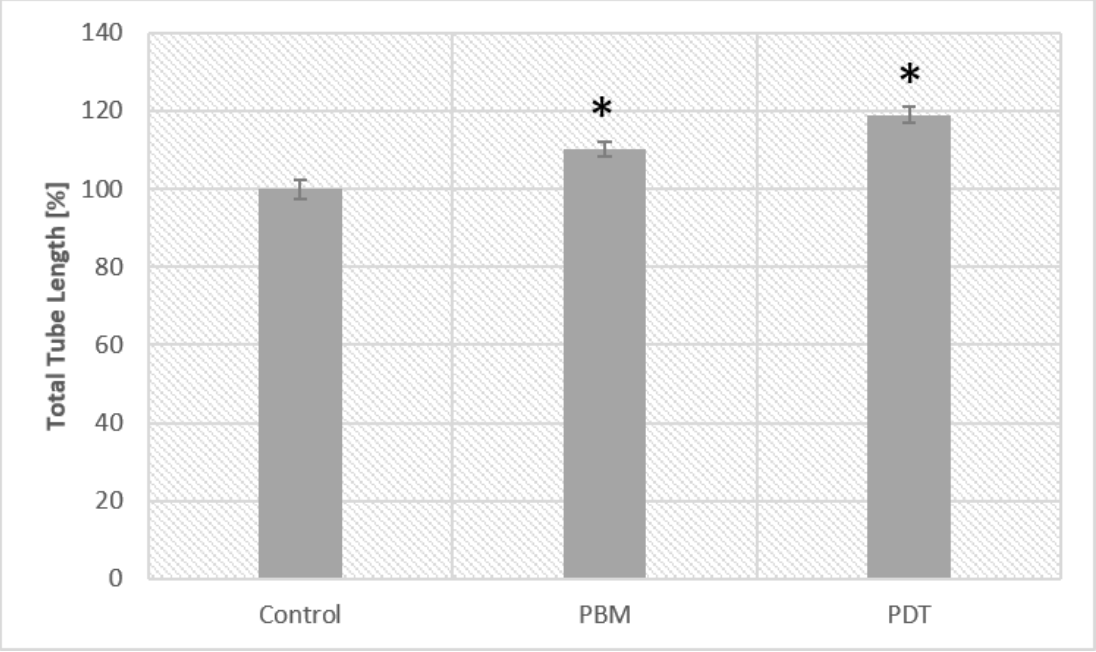

Figure 3.3d: The total length of the tubular structures in PBM and PDT triple treatment on day 7 (p<0.05, where * symbolizes significant differences compared to the control group)

3.4 Analysis of Intracellular ROS Generation

The ROS analysis shows that, surprisingly, PDT shows a clear drop after the first application, while PBM is fairly comparable to the control group and thus significantly higher than PDT. After the second light treatment, the value for PDT rises approximately by 60% compared to the value of the first day and is therefore similar to PBM and the control group. From the third light treatment onwards, an increase can be observed, this increase being higher in PDT than in the control group and PBM, although the value for PBM is barely higher than the control group as shown in Figure 3.4.

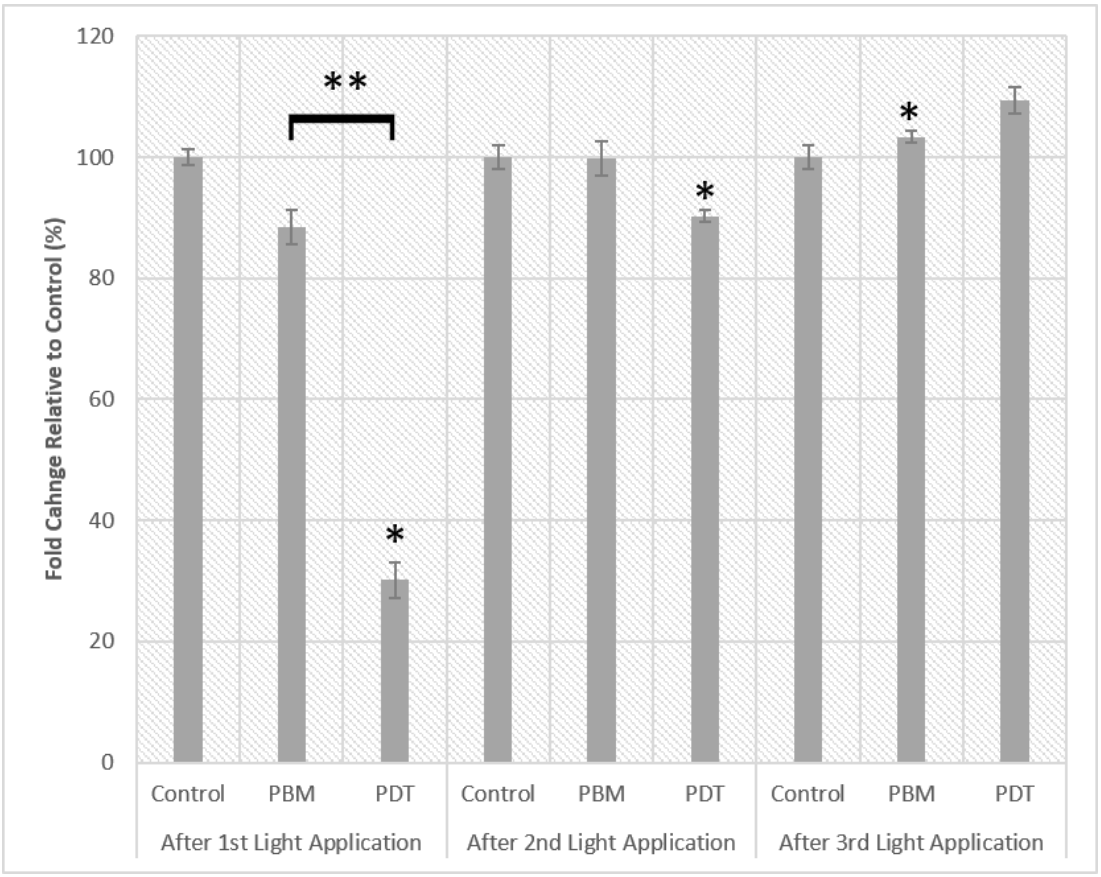

Figure 3.4: Intracellular ROS analysis in PBM and PDT groups after each light treatment (p<0.05, where * symbolizes significant differences compared to the control group and ** symbolizes significant differences between experimental groups).

3.5 Analysis of NO Release

As shown in Figure 3.5, the NO release in PBM after the first light application is nearly 4 times higher compared to the control group and PDT group. The NO release in PDT is comparatively as high as in the control group. After the second light application, surprisingly, the NO production in PBM drops drastically back down to half of the value of the control group, whereby NO release was visible in the PDT group. Compared to the value after the first application, NO release increased almost 5 times. After the last light application, the NO production in PBM increased to the control value level, whereby the NO release in the PDT group was almost the same as after the second light application.

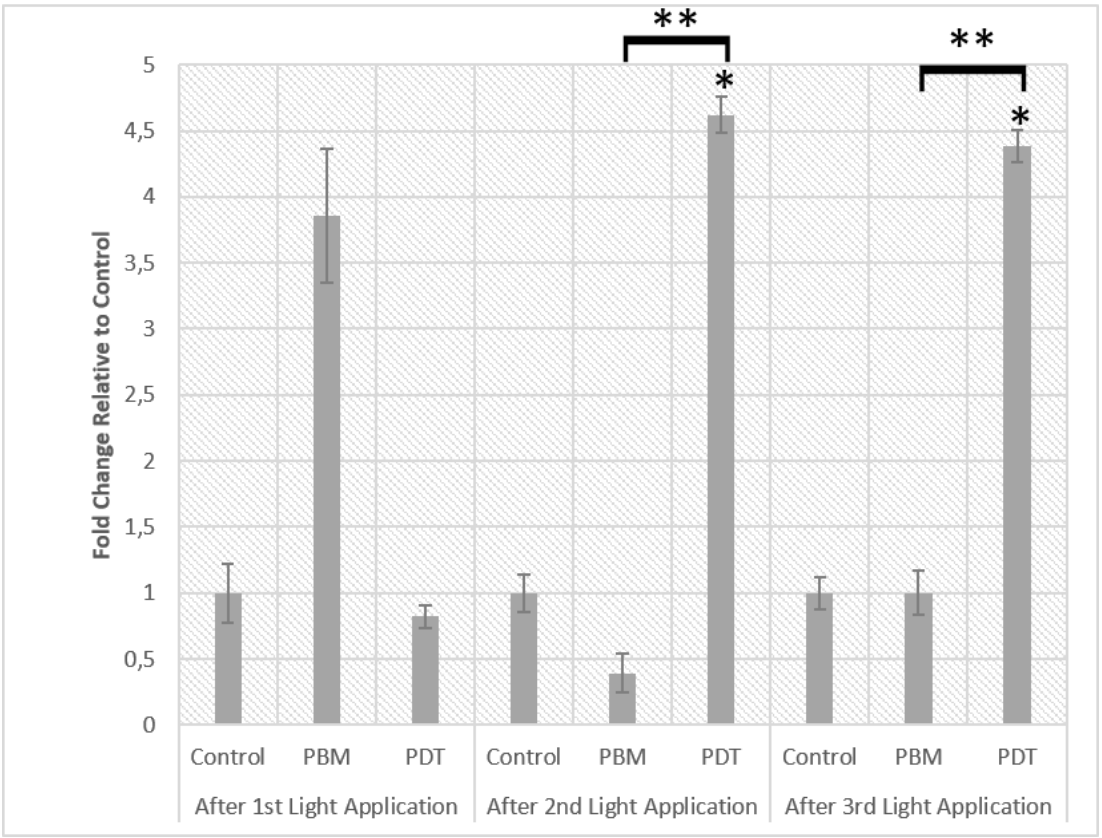

Figure 3.5: NO release in PBM and PDT groups after each light application ($p<0.05$, where * symbolizes significant differences compared to the control group and ** symbolizes significant differences between experimental groups).

3.6 Analysis of MMP change

The first day represents single and triple light treated groups since the laser was applied on the first day to all groups. On the microscope images shown in Fig. 3.6a, it can be observed, that the intensity of red fluorescence in every application is extremely high. The numerical data observed with a multimode reader by detecting the absorbance at 595-nm was given in Fig. 3.6b. On the first day, immediately after the light application, the detected MMP in PBM and PDT groups were almost the same. Both groups were approximately 15% higher compared to the control group. On the next day, the light application was performed only on triple treatment groups. In the single light treated groups, it was only added JC-1 probe to observe their MMP value day by day. The results showed that in PBM and also PDT in the single laser-treated groups the MMP value increases up to approximately 40% over the control value. The results after the second light treatment were surprisingly below the value of the single treated groups. PDT after second light treatment was slightly higher in value compared to PBM. On the third day, the value of the single treatments was observed without any other light application. The values were comparable with the values of the first day. PBM was approximately 5% higher compared to PDT.

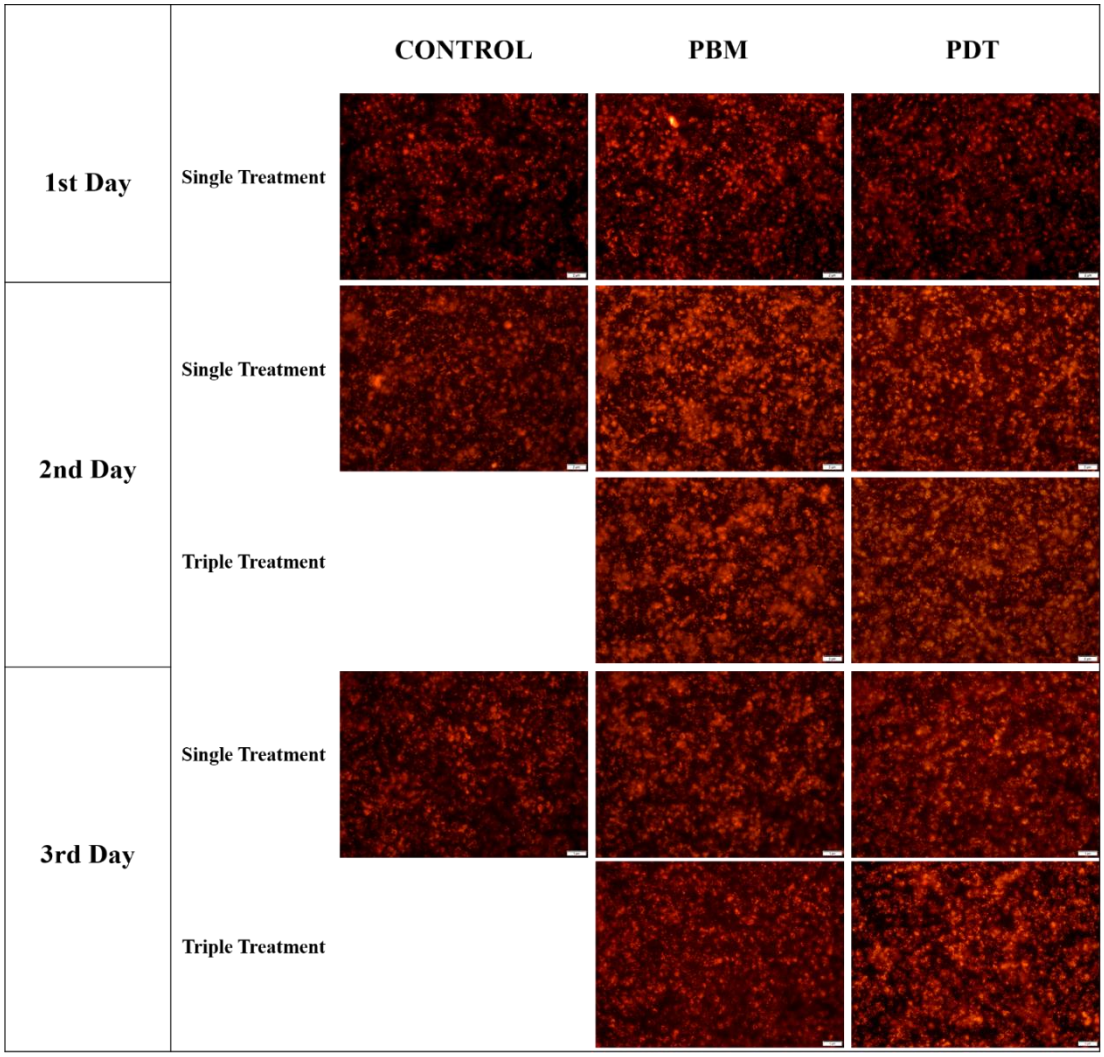

Figure 3.6a: Microscopic images of control, single and triple light treated PBM and PDT on first, second and third day. This image represents hyperpolarization of HUVECs at 595 nm (scale bar: 50 µm)

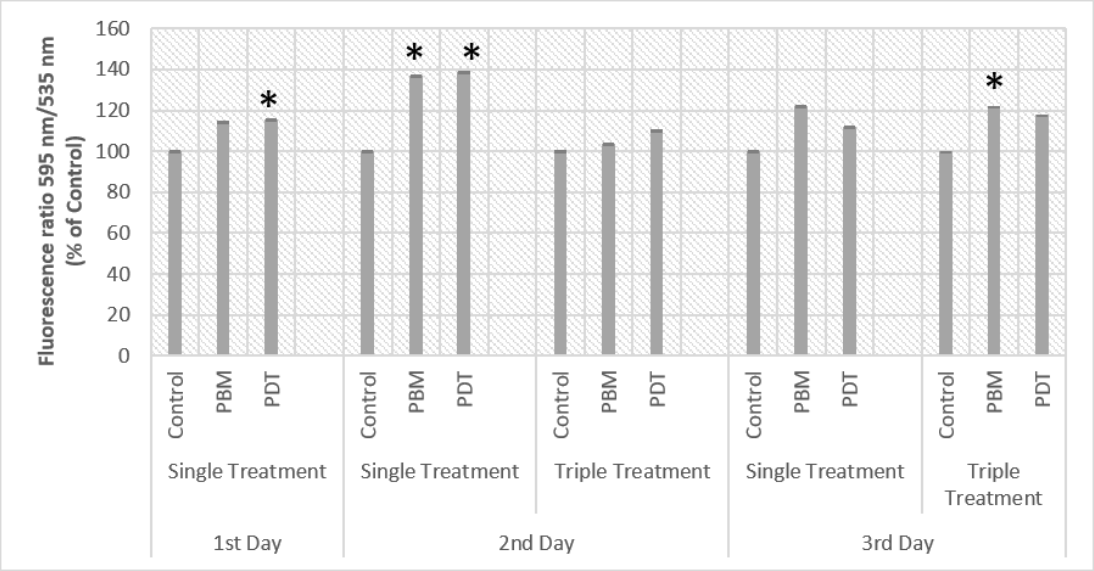

Figure 3.6b: Mitochondrial Membrane Potential Change after each light application in PBM and PDT groups day by day ($p<0.05$, where * symbolizes significant differences compared to the control group).

3.7 Immunofluorescent Staining

Immunofluorescence staining of HUVECs was done on day 7 after single and triple treatments were performed on both groups, PBM and PDT. The images of the proteins VEGF, PECAM1, and vWf, which have a red color, and the blue-colored DAPI staining, showing the nuclei of the cells, were captured with an inverted fluorescence microscope with 20X magnification.

The red color for each protein can be seen in each group, with different intensities in Fig 3.7. The control group was for all proteins moderated stained. Started with vWf, it can be observed, that the single treated groups were weaker in staining compared to the triple treated groups. The comparison between triple-treated groups with each other showed, that triple-treated low-dose PDT was significantly stronger than PBM. The expression of PECAM-1 showed better staining in the single treated PBM group compared to the single treated low-dose PDT group, whereas in triple treated PDT better and stronger staining was obtained than the PBM group. The strongest staining was achieved by the VEGF staining. Single treated PBM was strongly strained. In the triple treated groups, both PBM and PDT were expressed very strongly in the reddish

color. On closer consideration, however, it becomes clear that PDT delivers better results.

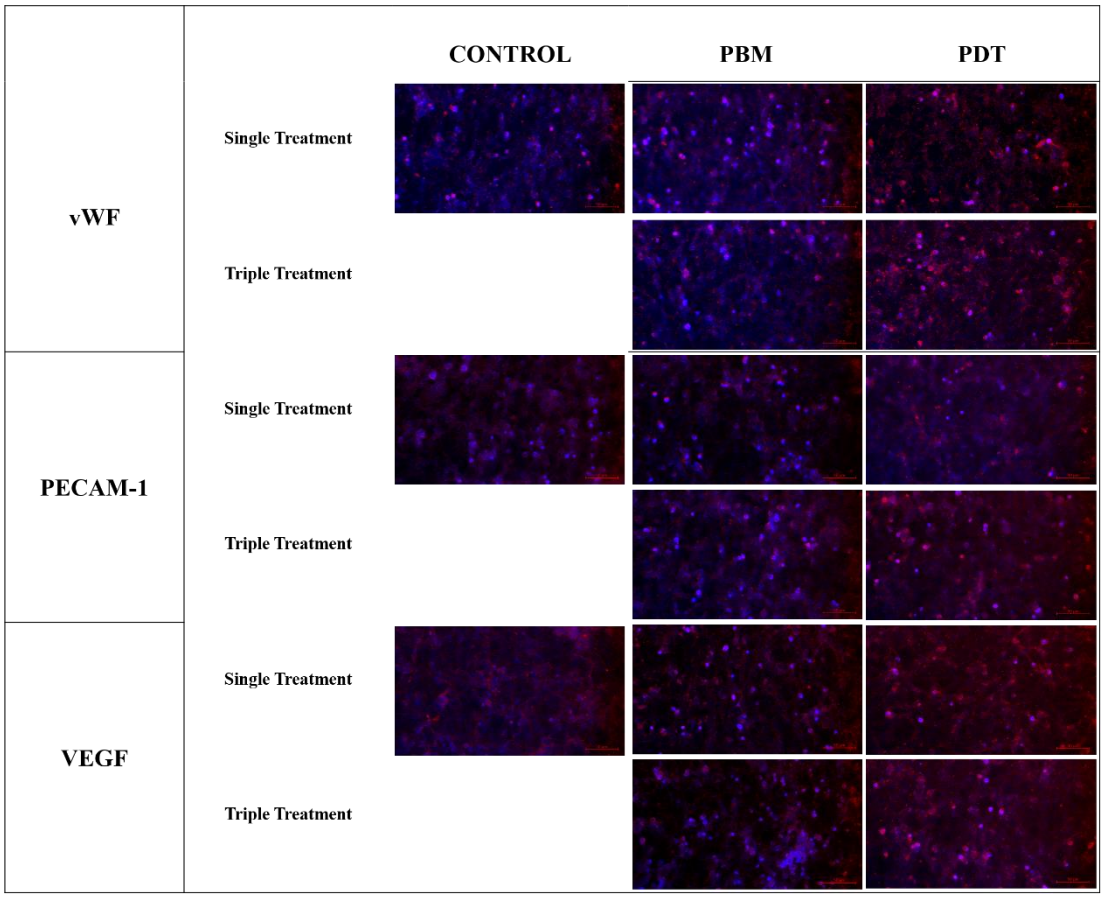

Figure 3.7: Immunofluorescence staining of control, PBM and PDT groups on day 7 with VEGF, PECAM-1 and vWf

3.8 Quantitative Real-Time PCR Analysis for the Expressions of vWf and PECAM-1 genes

Real-Time quantitative PCR was done on day 7 after single and triple light applications for each group of PBM and PDT. As shown in Figure 3.8, all groups resulted in higher expressions of vWf and PECAM-1 compared to the control group. Triple light treatment of PBM induced a higher expression for both genes than the single treatment of PBM did. Low-dose PDT application, whether was applied once or three times, induced higher expression of vWf than the single or triple treated PBM groups. For the gene PECAM-1, triple treatment of PBM showed an increased expression

compared to the single treatment of PDT group. For both genes, triple light treatment of PDT resulted in an overexpression compared to PBM and control group. Besides, none of these applications were statistically significant compared to the control group.

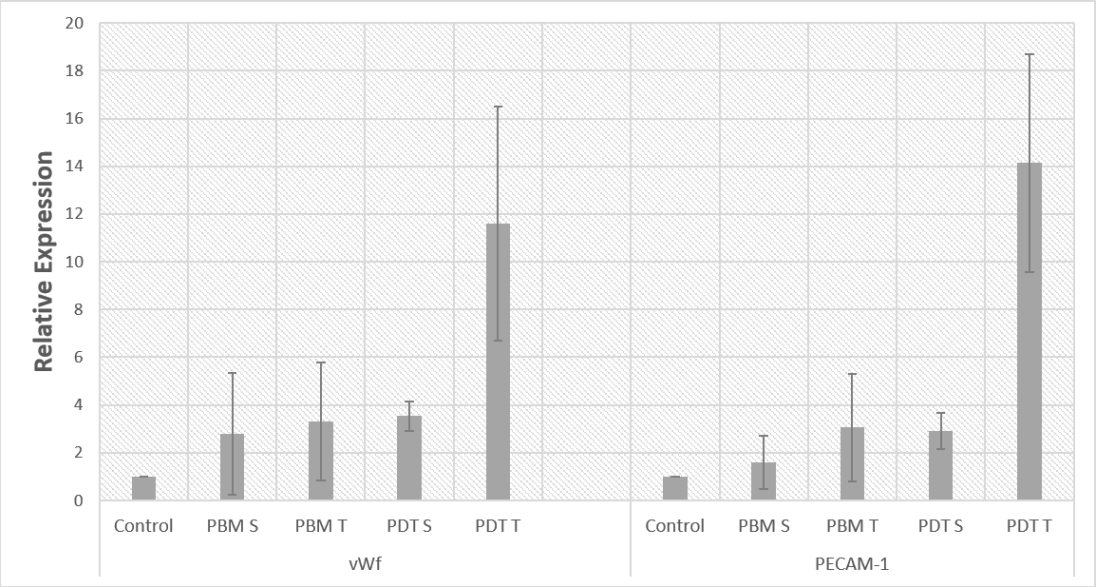

Figure 3.8: Real Time Quantitative PCR of control, PBM single treatment, PBM triple treatment, PDT single treatment and PDT triple treatment groups on day 7 of PECAM-1 and vWF.

3.9 Thermal Measurement

The thermal measurements showed only a little increase of approximately + 0.2°C during all light applications shown in Table 3.8. These results were expected because the light energy is absorbed by COX chromophores and a small part of this energy was transferred into heat energy. These results confirmed that the applications were non-thermal. A significant change in the heat would have side effects, which would have resulted in cell death. The combination of ICG and 808-nm light source brings heat increase with it. But with the regulation of the right doses and parameters of the light source and PS, this application of PDT was non-thermal and the safety was confirmed.

Table 3.9: Thermal Measurement during light applications.

	During 1st Light Application		During 2nd Light Application		During 3rd Light Application	
	PBM	PDT	PBM	PDT	PBM	PDT
Start (°C)	29,6	30	30,2	31	31,2	31
End (°C)	29,8	30,1	30,4	31,2	31,4	31,2
Δ t (°C)	0,2	0,1	0,2	0,2	0,2	0,2

Chapter 4

Discussion

This study aimed to show with the help of several analyses if PDT used in low doses has a biostimulative effect and compare it with PBM by using the same wavelength and energy density. First, it was important to obtain the appropriate concentration of ICG and to avoid any cytotoxicity on HUVECs, and also to determine the light parameters for this setup. Then the proliferation of the cells was detected with a cell viability test. Other analyzes were done to analyze intracellular ROS production, NO release, and MMP changes within the cells. Microscopic images were used for the comparison of the differentiation. And also, the immunofluorescence staining was done to compare the formation of the proteins, which are key factors for HUVECs for the proliferation and differentiation processes. In RT qPCR the gene expression of vWf and PECAM-1 was determined. All the experiments serve to determine whether and to what extent low-dose PDT has a photobiostimulative effect. The experiments were carried out to provide a better understanding of the respective mechanisms of both applications. Both mechanisms involve the production of intracellular ROS and NO release. Here, the effects of the respective proportions are brought into connection with the entire results.

First of all, it was important to start with a cytotoxicity test, because it has to exclude that ICG might have possible harm on the cells to be able to judge the results in a meaningful way. The results of cytotoxicity are roughly comparable. For this study, 0.1 µM was used, to exclude any possible side effects. At 0.5 µM there was a risk that the concentration might be too low to even get results in the form of differences, because it may be insufficient in terms of ROS production.

The importance of light sources and their parameters were explained with the help of the study of Topaloglu et al. using different laser sources with different energy densities and their effect on different cell types [12]. Another factor that shows the importance of the right parameters and the light source is shown with the different effects of high-dose PDT and the effect of low-dose PDT [44, 45]. The parameters must be optimized also because of the possible increase in temperature with the combination of 808-nm light source and ICG. High energy doses can cause heat energy, which leads to a photothermal effect. A study done by Chen et al. shows that the application of an 808-nm laser and ICG together results in a laser-tissue photothermal interaction [59]. To rule out the risk of overheating, the heat was measured during the irradiation. With a heat difference of approximately Δt 0.2°C, this was a positive result. Since a photothermal reaction can be ruled out, as there is a risk of a high increase of temperature, especially with 808-nm and ICG use in combination. The increase of temperature with the combination of 808-nm and ICG is because the light applications in NIR/IR region is normally photothermal and in this case, ICG is acting as a chromophore that strongly absorbs the photon energy. In this study, heat would be an undesired side effect. A body temperature is around 37°C, an increase of approximately 5-13°C, which equals to a temperature of approximately 42-50°C, can affect biological tissue thermally [60]. The result obtained during thermal measurement was an indication of photochemical effect. These results also show that low-dose PDT used with optimum light parameters can have a biostimulative/photobiomodulative effect. In summary, this result provides us with the fact that in this case there is no photothermal effect and a side effect due to the heat can be ruled out.

The microscopic images were obtained on days 1, 2, 3, 4, and 7. First and foremost, the images differ due to different cell numbers in comparison with each other. The reason for different cell numbers was the struggle with PDT washing procedures. The used cell number in PBM groups was $1x10^4$, whereas in PDT groups $1x10^5$ cells were used. The images generally show an increase in cell viability within day 1 to day 7 in both groups. One of the PBM effects in cell proliferation, which can also be observed in low-dose PDT. The comparison between single and triple light treatment showed that triple treatment has a more positive effect on cell proliferation in PDT groups compared to the single treated groups. On the other hand, it can be seen from the results

that PBM application induced better cell proliferation after a single treatment. The tubular structure formation provides further confirmation of these findings. On the images, a tubular structure can be observed. To provide this finding, ImageJ "angiogenesis analyzer" was used to obtain numerical data. The numerical findings were an indication of the differentiation of the HUVECs. After single light treatment, PDT gives poorer results than the control group, but PBM shows a better tubular structure formation compared to the control group and low-dose PDT group. This changes after triple treatment. With 18% above the control value, PDT provided a better tubular structure formation than PBM did, in which the increase was almost 10% higher than the control group. This can be explained by considering the increased kinetic of the tubular network structure during HUVEC culture in EGM due to the ROS formation leading to vasculogenesis. The results from MTT analysis and tubular structure formation suggested that cell proliferation and differentiation occurred, which reflected part of the PBM effects. Here you can see the real difference between high-dose PDT and low-dose PDT. Since PDT was traditionally used as a killing mechanism, but with the right parameters, this effect is used to do exactly the opposite.

Another important parameter is intracellular ROS production after light treatments. In non-toxic quantities, ROS act as signaling molecules to regulate biological and physiological processes [43]. ROS as the second messenger in pathways regulates proliferation and differentiation.

The results in ROS analysis were unexpected. It can be observed, that after a single light treatment the ROS production in low-dose PDT groups was extremely low. This value was below the control group. To eliminate any errors that occurred during the experiment, this experiment was repeated three times, each time leading to the same results. Surprisingly, this result in ROS did not affect other results, such as cell viability, differentiation, etc. to the extend. This result was unexpected since ROS is usually also produced in high-dose PDT to carry out the killing mechanism. The value in PBM after the first treatment was also below the control group but not to this extend. After the second irradiation, PBM and control group are comparable. In PDT, the ROS production value is still below the control value but has increased significantly compared to the results after the first light irradiation. From the third light irradiation, the value in PDT exceeds the other two groups. This ROS production has a positive

effect on cell proliferation and also on differentiation. From the last results of the ROS production, it can also be concluded that with low-dose PDT enough ROS was produced to have a biostimulative effect, but is still within limits. Because too much ROS production has a negative effect in the form of stress.

Another important factor is NO release in PBM applications. In this experiment, the NO value was measured with the help of the Griess reagent kit. The identical volume of Griess reagent and the supernatant solution from each sample was used to obtain the results. To be able to interpret the whole results better, these values also provide good clues.

After the first irradiation, there was an almost 4-fold difference in NO release in PBM groups compared to the control group. This result confirms the previous one since PBM in cell viability as well as in tubular structure formation always showed better results after a single treatment. The ROS values also confirm the entire obtained results. In PDT, on the other hand, the NO value was, compared to ROS, below the control value and therefore quite low. Surprisingly and unexpectedly, the value in PBM drops rapidly after the second irradiation and only reaches half the control value, with PDT rising to just under five times the control value. After the third irradiation, the NO value in PBM rises to the level of the control value, but PDT remains relatively stable and hardly changed. Given all the results in comparison, it can be concluded that precisely these increases in ROS and NO may have a positive effect on cell proliferation and cell differentiation. Besides, it was understood that the mechanism of action depends on these molecules which are responsible to initiate several signaling pathways finally result in cell proliferation and differentiation.

Light is absorbed by COX causing the separation and release of NO. The electrons are subsequently transferred up the respiratory chain as oxygen takes its place. This results in a proton gradient across the mitochondrial membrane, which causes changes in MMP, ATP, and intracellular ROS generation. The MMP changes in this study were obtained by the JC-1 probe on day 1, day 2, and day 3, for single treatment groups and triple treatment groups. Hyperpolarization of the mitochondria is induced by PBM and PBM-like processes, and depolarization expresses cell death. The microscopic images in figure 3.6 show that cell viability was very high in all groups, as red represents the living cells. To be able to assess the results of the MMP analysis more precisely, the

numerical datasets must be taken into account. The MMP results obtained after the first light application was for PBM and PDT comparable. Surprisingly on the second day of the experiment, data obtained for single treatment groups, which were only irradiated with laser on the first day, had an increased MMP value compared to the control group. The result obtained for PDT after second light irradiation was slightly higher in value compared to PBM. After the third light treatment, PBM was approximately 5% higher compared to PDT. The differences were not that big. Compared to the other results, one would have expected a higher value in PBM after the first irradiation and in PDT after the third irradiation. But since MMP was only one part of the PBM mechanism, it has not affected the other results as much. The almost comparable results confirm again that low-dose PDT has a biostimulative effect.

Although single treatment results in PBM are better than in single treated PDT, the results in MMP are relatively close and comparable. This can be related to the fact that COX in PBM is the chromophore that absorbs light, while in PDT the light is absorbed by an exogenous molecule, in this case, the PS was ICG. For this reason, the values in MMP change in PBM are comparable to the results achieved in low-dose PDT.

The cell proliferation and differentiation capacity of endothelial cells are part of vascularization and also angiogenesis. For therapeutic uses, such as wound healing vascularization of endothelial cells is important [61]. HUVECs form tubular structures by expressing cell markers such as vascular endothelial growth factor (VEGF), Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), and von Willebrand factor (vWf) [62, 63]. The effect of PBM with 660 and 780 nm laser on the gene expression of three angiogenic markers including VEGF was enhanced [64]. Another study on human keratinocyte cells irradiated with 780 nm wavelength increased VEGF [65]. We can confirm these studies with our results.

Immunofluorescence staining using vWf, PECAM-1, and VEGF was captured with 20X magnification on the 7^{th} day. HUVECs showed stronger staining compared to the control group after light irradiation. The strongest staining was revealed in VEGF. VEGF is one of the most important factors during angiogenesis. The results of strong straining were expected since VEGF leads endothelial cells to vascularization by accelerating the proliferation and also differentiation capacity of the cells. The microscope images show that triple-treated PDT in low doses and PBM had

comparable results. The results in vWf showed, that in general, triple treated groups were stronger strained compared to the single treated groups. In PECAM-1 stained groups, in single treated groups PBM was stronger and in triple treated groups low-dose PDT. In general,

The moderated staining in control was due to the growth medium used for cell culture. EGM contains different growth factors, which promote also without any light treatment those protein expressions. VEGF is a proliferation stimulating gene, which explains its significantly remarkable strong staining. The increase in cell viability and differentiation, observed in form of tubular structure formation length can be compared with the results in VEGF expression. Since it enhances endothelial cells proliferation and differentiation. VEGF action is known to be determined by VEGF binding to its membrane receptors. The activation of endothelial cell division results in receptors being used up. Light irradiation also leads to changes and activities in the membrane in the form of ROS production, NO release, and MMP changes. These changes can affect the binding of the integrins to the proteins.

The vWf expression is needed for vascularization processes in endothelial cells and is a key factor in terms of proliferation. A deficiency in vWf may lead to vascular damage or it can cause von Willebrand disease, which is the most prevalent inherited bleeding illness [66]. The results in cell proliferation, differentiation, and also immunofluorescence images indicate that enough vWf was produced to promote proliferation and vascularization and not to damage the cells or cause any unwanted side effects.

The RT qPCR results confirm with the results of other assays, since PDT after triple treatment resulted in overexpression in vWf and PECAM-1. In ROS production, NO release, cell viability and tubular structure formation triple treated PDT results higher compared to PBM and control. Immunofluorescence staining of those genes resulted also in strong stained triple treated PDT.

In summary, it can be observed that low-dose PDT had a biostimulative effect. In terms of cell proliferation and cell, viability triple treated PDT was even more effective than PBM. Further analyzes such as ROS and MMP after triple treatment had almost comparable results in both groups. Only NO had a significantly higher release in low-

dose PDT than in PBM after the triple treatment. With the temperature measurement, a rise in temperature with unwanted side effects was also excluded. The immunofluorescence staining recordings confirm the previous results, as both groups showed comparable results.

Chapter 5

Conclusion

First and foremost, this study confirms the assumption that low-dose PDT has similar and comparable effects to PBM. A closer look reveals that in most analyzes, PBM generally delivers slightly better results than low-dose PDT after a single treatment. However, PDT still delivers better results than the control group after a single treatment in most analyzes. The results after triple treatment are decisive here. PDT not only confirms the assumption that it has a similar effect to PBM in low doses, but also shows better results, especially in cell proliferation, cell differentiation, and protein expression.

In this study, we tried to compare the effect of low-dose PDT with PBM using 808-nm of wavelength by several analyzes. The analyzes that were carried out provided good clues, but some questions remain open. The results obtained in the analysis of intracellular ROS generation are still questionable. ROS is a key factor in PBM and affects cell proliferation, differentiation is needed to make the mechanism work properly. After the first day of PDT treatment, the value of PDT is very low, but it was achieved cell proliferation and also differentiation of PDT anyway.

For further studies, we aim to determine the total protein amount by using the lowry method or BCA method. Vascularization and angiogenesis of complex tissues and also organs are essential in regenerative medicine. This study offers an opportunity for tissue engineering to improve and may accelerate the process of wound healing, vascularization of implanted tissue since vascularization is a challenge in this field. *In vivo* studies on larger series with different application parameters and PS,

concentrations would provide more effective results for the future perspectives and possible uses in clinical application.

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Curriculum Vitae

Name Surname : Dilara Portakal Koç

Education:

2014–2018	Hochschule RheinMain (Germany), Dept. of Interdisciplinary Eng.
2019–2021	İzmir Kâtip Çelebi University, Dept. of Biomedical Eng.

Republic of Turkey

İzmir Kâtip Çelebi University

Graduate School of Natural and Applied Sciences

Possible Biostimulative Effect of Low-Dose Photodynamic Therapy on HUVEC Cells

Department of Biomedical Engineering

Master's Thesis

Dilara Portakal Koç

ORCID 0000-0001-8043-712X

Thesis Advisor: Dr. Nermin Topaloğlu Avşar

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