PHOTOBIOMODULATION ON HUMAN OSTEOBLASTS AND OSTEOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS

by

Gamze Bölükbaşı Ateş

B.S, in Physics, Boğaziçi University, 2005

M.S, in Biomedical Engineering, Boğaziçi University, 2007

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APPROVED BY:

Prof. Dr. Murat Gülsoy ..... (Thesis Advisor)

Assoc. Prof. Dr. Bora Garipcan ..... (Thesis Co-advisor)

Prof. Dr. Ata Akın .....

Prof. Dr. İnci Çilesiz .....

Prof. Dr. Can Yücesoy .....

Assist. Prof. Dr. Özgür Kocatürk .....

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To my beloved family...

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ABSTRACT

PHOTOBIOMODULATION ON HUMAN OSTEOBLASTS AND OSTEOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS

The present *in vitro* comparative study evaluated parameters of osteogenesis under the influence of photobiomodulation (PBM). PBM uses light in the visible and near-infrared spectrum to induce a non-thermal process and to activate endogenous chromophores, which may result in therapeutic outcomes. Although the cellular and molecular mechanisms involved in the PBM are still unclear, studies suggest that reactive oxygen species (ROS) produced in response to PBM, can induce activation of many biological pathways. Adipose-derived stem cells are promising for use in regenerative medicine and promoting their osteogenic differentiation would be used in improving bone tissue healing and regeneration.

The effects of PBM at two different wavelengths with three different energy densities on human osteoblasts and osteogenic differentiation of adipose-derived stem cells were investigated in this present study. Another purpose of the study was to associate the possible biostimulative effect of light with photosensitizers (PSs), which are light activated molecules that cause ROS generation. The cells were incubated with Indocyanine Green (ICG) and Methylene Blue (MB) prior to laser irradiation.

Assays measuring the cell viability, cell proliferation, alkaline phosphatase activity (ALP), mineralization, ROS generation and osteoblast specific gene expressions were performed. The results of the present study showed that combined light and PS treatment does not result in a synergistic enhancement of PBM on cell viability and proliferation, but detailed analysis revealed that mineralization and ALP activity were altered following only light or photosensitizer mediated light applications. Whether biostimulative or inhibitory effect occurs after PBM and PS-combined PBM depends upon light dose and wavelength. The potential applications of PBM may be numerous but adequate and reliable evidence is necessary to recommend PBM for clinical use.

Keywords: Photobiomodulation, osteoblasts, osteogenic differentiation, adipose-derived stem cells, low-level laser light

ÖZET

İNSAN OSTEOBLAST KEMİK HÜCRELERİ VE YAĞ-DOKU KÖK HÜCRELERİNİN OSTEOJENİK FARKLILAŞMASI ÜZERİNDE FOTOBİYOMODÜLASYON ETKİLERİ

Bu karşılaştırmalı *in vitro* çalışmada kemik oluşumunda fotobiyomodülasyon etkileri araştırılmıştır. Fotobiyomodülasyonda görünür ve yakın kızılaltı ışığın ısıl olamayan bir etki yaratıp hücre içinde bulunan kromoforları etkileyerek tedaviye yönelik sonuçlara neden olabildiği düşünülmektedir. Fotobiyomodülasyonun moleküler ve hücresel aktiviteler üzerindeki etkisi tam olarak açıklanamasa da oksijen radikallerinin biyolojik yolakları etkinleştirdiği önerilmektedir.

Bu çalışmanın bir amacı iki farklı dalgaboyu ve üç farklı enerji yoğunluğunda ışığın insan osteoblast kemik hücreleri ve yağ-doku kök hücrelerinin osteoblasta farklılaşması üzerindeki etkilerini incelemektir. Çalışmanın diğer amacı ise ışığın muhtemel uyarıcı etkisinin ışığa duyarlı maddeler ile arttırılmasının incelenmesidir. Hücreler ışık ile uyarılmadan önce İndosiyanine yeşili ve Metilen mavisi ile inkübe edilmişlerdir.

Hücre canlılığı, hücre çoğalması, alkalin fosfataz aktivitesi, mineralleşme seviyesi, oksijen radikallerinin oluşması ve kemik hücresine dair gen ekspresyonlarını ölçmek üzere testler yapılmıştır. Çalışmanın sonuçlarına göre ışık ve ışığa duyarlı maddenin birlikte uygulanması hücre canlılığı veya çoğalmasında değişiklikler yaratmasa da, detaylı incelemelerde mineralleşme ve alkalen fosfataz üretiminde tek başına veya ışığa duyarlı madde ile birlikte uygulanan ışığın etkilerinin olduğu gözlemlenmiştir. Işık ve ışığa duyarlı madde ile uygulanan ışığın etkisinin biyostimülatif veya biyoinhibitör olması ışığın dalgaboyuna ve dozuna bağlıdır. Fotobiyomodülasyonun gelecek vaat eden çok çeşitli uygulamaları olabileceği düşünülse de klinik uygulamalardan önce uygun ve güvenilir parametrelerin belirlenmesi gerekmektedir.

Anahtar Sözcükler: Fotobiyomodülasyon, osteoblast kemik hücresi, osteojenik farklılaşma, yağ-doku kök hücre, düşük dozda laser uygulaması

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LIST OF ABBREVIATIONS

AB	Alamar Blue
ALP	Alkaline Phosphatase
ALPL	Alkaline phosphatase gene
ANOVA	Analysis of Variance
AO	Acridine Orange
ASC	Adipose-derived stem cells
BGLAP	Osteocalcin gene
BMP	Bone morphogenetic protein
cDNA	Complementary DeoxyriboNucleic Acid
COL1A	Collagen gene
DMEM	Dulbecco's Modified Eagle's Medium
ICG	Indocyanine green
MB	Methylene Blue
MSC	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
ODM	Osteogenic differentiation medium
PBM	Photobiomodulation
PBS	Phosphate-buffered saline
PDT	Photodynamic therapy
Pi	Inorganic phosphate
PI	Propidium Iodide
PS	Photosensitizer
RNA	RiboNucleic Acid
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
RUNX-1	Runt-related transcription factor 1

1. INTRODUCTION

1.1 Motivation

Light has been investigated as a therapeutic and diagnostic tool for decades in medicine and dentistry. Photobiomodulation (also known as low level laser therapy) uses low-level laser light (visible/near infrared region) in order to induce cell proliferation, tissue regeneration, wound healing [1], reduces inflammation [2] and pain [3] by modulating mitochondrial activity and respiratory chain reactions [4]. However the desired effect depends on many parameters such as irradiation time, wavelength, power, density as well as tissue or cell types, their conditions. Although the first preliminary studies in the literature on PBM were promising, conflicting results are present in the literature and the exact underlying mechanisms have not been fully understood yet. This biphasic dose response of light may also be a cause of inconsistent reports on PBM.

PBM, which is an easy and noninvasive method, has a potential to have a beneficial impact in bone tissue engineering and in regenerative medicine. Clarifying mechanisms in osteoblast biology may be the first step in treating bone related diseases such as osteoporosis, osteogenesis imperfecta, Paget's disease, fibrous dysplasia, and many others [1].The multilineage capacity and ease of isolating large quantities of ASCs makes them promising candidates for stem cell therapy for which exploring alternative methods to increase cell proliferation would be helpful.

Photosensitizers (PS) have also been used in clinical applications like photodynamic therapy (PDT). In PDT laser energy is combined with a photosensitizer (PS) to induce the production of singlet oxygen and free radicals, which will react immediately with oxygen (O_2) and start a cascade of reactions, which will cause damage the target tissue, or living agents like bacteria or microbes. However, it has been recently shown that PS applications can also have proliferative effects on wound healing process [2–4].

The question is that whether the combined PBM and PS application would have a synergetic effect on cell viability/proliferation/differentiation or possible biostimulative effects would be annihilated. A detailed research of PBM and PS-mediated PBM on osteoblasts and osteogenic differentiation of stem cells *in vitro* would provide valuable information for further *in vivo* studies and undoubtedly contribute to better understand the potential benefits of PBM.

1.2 Objectives

The aim of this study is to analyze the effect of PBM and the use of PSs on the behavior of human osteoblasts and adipose-derived stem cells (ASCs) differentiation into osteoblasts. The following specific aims are considered in this research:

- To investigate the effect of visible red laser irradiation at different energy densities on human osteoblast cells *in vitro*,
- To investigate the effect of near infrared laser irradiation at different energy densities on human osteoblast cells *in vitro*,
- To determine non-toxic methylene blue (MB) and indocyanine green (ICG) concentration for osteoblast viability *in vitro*,
- To investigate the effects MB-mediated visible red laser irradiation at different energy densities on human osteoblast cells *in vitro*,
- To investigate the effects ICG-mediated near infrared laser irradiation at different energy densities on human osteoblast cells *in vitro*,
- To determine non-toxic MB and ICG concentration for ASCs viability *in vitro*,
- To investigate the effect of visible red laser irradiation at different energy densities on differentiation of ASCs into osteoblasts *in vitro*,
- To investigate the effect of near infrared laser irradiation at different energy densities on differentiation of ASCs into osteoblasts *in vitro*,

- To investigate the effects MB-mediated visible red laser irradiation at different energy densities on differentiation of ASCs into osteoblasts *in vitro*,
- To investigate the effects ICG-mediated near infrared laser irradiation at different energy densities on differentiation of ASCs into osteoblasts *in vitro*,
- To compare the effects of visible and near infrared laser irradiation on osteoblasts and ASCs differentiated into osteoblasts *in vitro*.

1.3 Outline

Chapter 1 is the introduction in which motivation and the objectives of the research are listed.

Chapter 2 includes background information on bone tissue, cells types that are involved in osteogenesis and ASCs differentiation processes. Basic principles and mechanisms on PBM are explained. Finally, the properties of photosensitizers, detailed information about MB and ICG are introduced.

In Chapter 3, all experimental design and protocols are explained.

Chapter 4 gives and discusses the results of 8 separate studies and ends with a discussion of parameters that affects the results.

In Chapter 5, the outcomes of the research are summarized and conclusions are stated.

2. BACKGROUND

2.1 Bone Tissue

Bone is a form of connective tissue. The extracellular matrix, which makes up 90% of the volume of the tissue, separates the cells. Bone matrix weight is made of 35% organic materials, 60% inorganic materials and 5% of water [5]. The inorganic materials, which are mainly mineral salts such as calcium and phosphate, give bone its stiffness and its strength to support without bending. Bone mineral crystals are classified as apatite, which contain both carbonate ions and acid phosphate groups. The organic part of the matrix is composed of 90% of collagen (predominantly Type I) and 10% of non-collagenous organic materials, which are composed of endogenous proteins (osteocalcin, osteonectin, bone sialoprotein etc.) produced by the bone cells. The organic matrix gives bones tensile strength and flexibility.

Bone is made up of two types of tissues; compact and spongy bone. They differ in density. Spongy bone tissue has a 50-90% porous structure therefore its strength is lower compared to compact bone tissue. Compact bone tissue, which is hard and dense, includes only osteocytes, canaliculi and blood vessels and provides protection and support.

Four types of cells constitute the bone tissue: osteoblasts, osteocytes, bone lining cells and osteoclasts (Figure 2.1).

a) Osteoblasts: Mesenchymal stem cells are precursors for pre-osteoblasts, which differentiate into osteoblasts. They are fibroblast-like cells which have cuboidal shape when they are in active form. Osteoblast activity leads to bone formation and mineralization. They synthesize type I collagen and non-collagenous proteins and form the extracellular matrix which will be calcified with inorganic salts.

b) Osteocytes: Once the osteoid (the bone matrix that forms prior to the maturation of bone tissue) surrounding an osteoblast is completely calcified, the cell is called an osteocyte. They are mature bone cells, no longer capable of cell division, which are thought to control the activity of osteoblasts and osteoclasts. Osteocytes are connected to each other via long cytoplasmic extensions. As the osteoblast transitions to an osteocyte, alkaline phosphatase is reduced, and osteocalcin is elevated.

c) Osteoclasts: These large multinucleated cells are from the monocyte-macrophage cell line. Their role is to remove the mineralized matrix and break up the organic matrix rich in collagen fibers. As resorption occurs, osteoblasts in turn, they form new bone. Equilibrium between osteoblasts and osteoclasts maintains bone tissue. Each resorption by osteoclasts is followed by a bone formation phase by osteoblasts. When the resorption is terminated, the released proteins attracts osteoblast bone formation will start.

d) Bone Lining Cells: They are flat and elongated cells which completely line the surface. Osteoblasts become bone lining cells but they may be reactivated and function as a barrier for certain ions and any osteoclast resorptive activity. Bone-lining cells are thought to regulate the movement of calcium (Ca^{2+}) and phosphate (P) into and out of the bone.

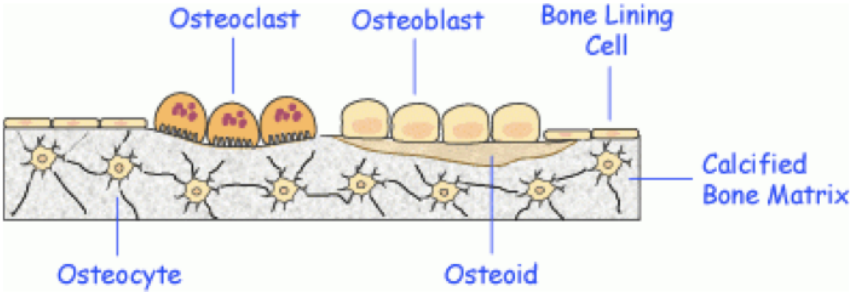

Figure 2.1 Schematic view of bone cells [6].

Osteogenesis is the bone development which can occur by ways: intramembranous and endochondral [7]. In the process of endochondral bone formation, mesenchymal stem cells (MSCs) start to differentiate into chondrocytes and secrete a cartilaginous matrix, which will be later replaced by bone. Both long and short bones in the body are formed this way. In intramembranous ossification, instead of forming cartilaginous matrix, MSCs

differentiate directly into osteoblasts. This type of ossification forms flat bones. The growth in diameter of bones around the diaphysis (midsection of a long bone) occurs by appositional growth, which is the deposition of bone beneath the periosteum (outer surface membrane).

Bone modeling and remodeling realizes during different stages of life. Modeling occurs when bone resorption and bone formation realize at different parts of the bone. This process is seen during birth to adulthood. On the other hand, bone remodeling is used to replace of old tissue by newer one, which is needed mainly in the adult skeleton to maintain bone mass.

2.2 Adipose-derived Stem Cells (ASCs)

Mesenchymal stem cells are considered as one of the most promising cell types for therapeutic applications. They were originally identified in bone marrow and considered to be multipotent [8].MSCs can be easily isolated, expanded and have low immunogenicity, have potential for long-term generation. Therefore they receive attention to develop methods for repairing bone defects in regenerative medicine. MSCs have been isolated from adipose tissue [9], umbilical cord blood [10], peripheral blood [11], dental pulp [12], dermis [13], and amniotic fluid [14].

Adipose-derived stem cells (ASCs) were first identified as MSCs in adipose tissue in 2001 [15]. In literature, there exist several names for adipose-derived stem cells such as Adipose Derived Adult Stem (ADAS) Cells, Adipose Derived Adult Stromal Cells, Adipose Derived Stromal Cells (ADSC), Adipose Stromal Cells (ASC) and Adipose Mesenchymal Stem Cells (AdMSC). However, in 2004, the term as Adipose-derived stem cells (ASCs) has been adopted in a consensus.

ASCs are preferred among other MSCs because they are abundant in the human body. They can be isolated from subcutaneous adipose tissue in high numbers. ASCs can be differentiated into multiple lineages such as adipogenic, chondrogenic and osteogenic

cells under culturing with specific conditions [15] . The ostegenic media should contain 1 nM dexamethasone, 2 mM β-glycerolphosphate and 50 μM ascorbate-2-phosphate [16]. The cells differentiate into osteoblast-like cells in 2 to 4 weeks. The osteogenesis can be monitored by analysis of key osteogenic genes such as alkaline phosphatase Type I collagen, osteoponin, osteocalcin, bone sialoprotein, Runx-1, BMP-2, BMP-4, parathyroid hormone receptor etc [17]. Osteogenesis by human ASCs, together with other animals such as rabbits, dogs, horses, rhesus monkey have been studied [18].

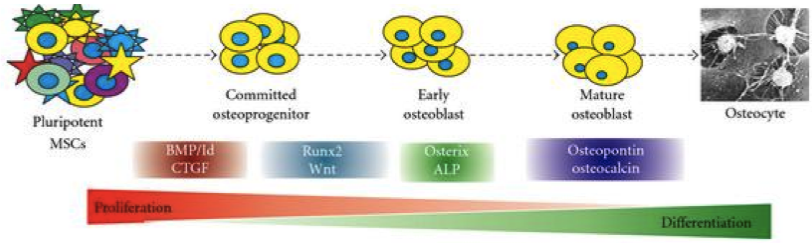

Figure 2.2 Schematic of osteogenic differentiation of MSCs. The differentiation of ASCs into osteoblasts is controlled by different kinds of morphogens, hormones, growth factors, cytokines, and ECM proteins [19].

Osteoblast differentiation (Figure 2.2) i*n vitro* and *in vivo* can be characterized in three stages:

1. cell proliferation,

2. matrix maturation,

3. matrix mineralization .

All stages are controlled by several transcription factors. During proliferation, they express several extracellular matrix proteins such as fibronectin, collagen, TGF-β receptor 1, and osteopontin. Alkaline phosphatase and collagen expressions are higher in the matrix maturation stage. In the third stage, genes for proteins such as osteocalcin, BSP, and OPN are expressed and deposition of mineral substance is promoted [1].

2.3 Photobiomodulation

Photobiomodulation (PBM) has been used in different branches of medicine and dentistry for the last tree decades in pain and inflammation reduction [1], in tissue repair [2], as well as to promote regeneration and to accelerate wound healing [3] . PBM is the application of red or near infrared coherent (lasers) or noncoherent (LED) light with an output power 1-500 mW and with low energy densities compared to other laser therapy forms such as ablation or coagulation which are heat-mediated methods [4]. Laser light is delivered to the target tissue or cell layer in continuous or pulsed mode. At low doses, energy densities delivered to the target tissues do not emit heat, sound or vibrations. So the temperature increase is negligible and does not cause any thermal damage to the target tissue. Schneede et al. has reported a temperature increase of less than 0.065oC with laser irradiation of 40 mW/cm^2 [20]. The transmitted energy does not cause any thermal effect but induces photochemical reactions that will modulate cellular metabolic processes. Cellular parameters that are examined include cell numbers, cell viability, proteins synthesis, DNA damage, cell morphology and RNA expressions.

The biological mechanisms related to the biostimulative effects of laser irradiation are not fully understood. It appears that PBM has a wide range of effects at the molecular, cellular, and tissue levels. One theory claims that the laser energy is absorbed by the primary cellular chromophore: cytochrome c oxidase. It is located in the inner mitochondrial membrane and it is the last enzyme in the respiratory electron transport chain. The absorbed energy is converted to the metabolic energy because ATP levels increase after laser irradiation. At the same time, release of reactive oxygen species from the electron transport chain activates transcription factors. An increased ATP level increases protein synthesis, DNA synthesis and expression of growth factors and cytokines resulting in high rate of cellular proliferation [21].

The primary reactions after light absorption are reactions that occur because of the alterations in photoacceptor function. The secondary reactions are cellular signaling pathways. The suggestions about the primary mechanisms of light action can be summarized as:

• Singlet-oxygen hypothesis: the photo-acceptor generates singlet oxygen when it absorbs light and this production stimulates RNA and DNA synthesis rate.

• Redox properties alteration hypothesis: altered redox state of chromophores in the cytochrome c oxidase molecule via photoexcitation consequently, increases the rate of electron flow in the molecule.

• Nitric oxide (NO) hypothesis: the activity cytochrome c oxidase of is partially inhibited by NO to slow respiration in order to direct O_2 elsewhere is necessary. Irradiation may photodiassociate NO from its binding sites and in this way increases the O_2-binding and respiration rate.

• Transient local heating hypothesis: a small part of the absorbed energy is converted to heat, which will cause local transient rise in temperature. This increase may cause structural changes and trigger biochemical activity.

• Superoxide anion hypothesis: depending on the metabolic state of mitochondria, production of superoxide anions can be increased after light irradiation.

The redox state of the mitochondria is altered in all of these mechanisms, leading to increased oxidation, but the light dose and intensity used may cause domination of one of the mechanisms (Figure 2.3).

Although there are several studies investigating PBM (previously called Low Level Laser Therapy/LLLT), the results are controversial. The ideal doses, time of exposure, the proper wavelengths to induce favorable cell responses have not yet been defined. Different experimental procedures, cell types and timings of irradiations results in different and conflicting outcomes.

A biphasic dose response of PBM has been seen in the studies. PBM produce a better result at low doses than high doses of the same wavelength. If the energy delivered is insufficient, there is not any response because the minimum threshold is not reached

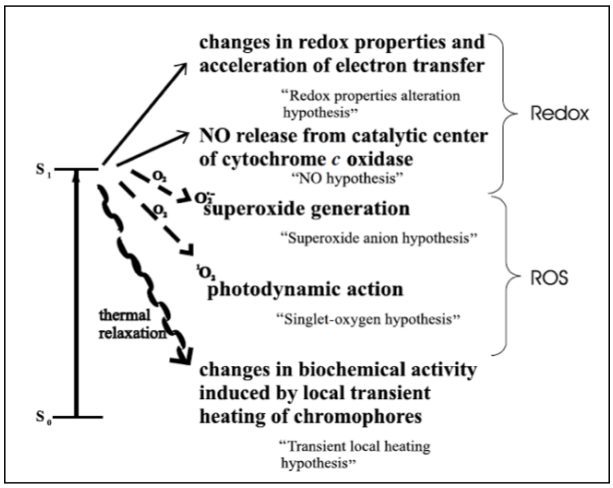

Figure 2.3 The suggested hypothesis on the primary biochemical reactions action after light absorption [22].

yet. However, after the threshold, if more energy is applied, a negative result is obtained causing bioinhibition instead of biostimulation [23] .

2.4 Photosensitizers

2.4.1 Stimulation of Photosensitizers

Photosensitizers are photon-absorbing molecules that can be found endogenously in the cells or they can be taken up from exogenous sources. Most molecules that absorb light finally lose this energy by radiationless decay (internal conversion). However, instead of internal conversion, electron or energy transfer to other molecules like molecular oxygen occurs in PS molecules.

The PS is excited to a singlet-excited state S_n from the ground state S_0 when it absorbs the photon energy. Then, the PS may relaxate to the lowest excited singlet state S_1. Intersystem crossing may generate the PS triplet state T_1. T_1 excited state of the PS, having longer lifetime than S_1 state, reacts in two ways; Type I mechanism and Type II mechanism. Whether type I and type II reactions will occur depends on the presence or absence of singlet O_2 (Figure 2.4).

In the Type I mechanism, PS reacts with an electron donating substrate. As a result free radicals are produced. These radicals can react with oxygen to form superoxide anion radicals.

In the type II mechanism, the activated PS can transfer its energy directly to oxygen, to form singlet O_2, which is a highly reactive oxygen species (ROS). Singlet O_2 can oxidize substrates that are unaffected by O_2 in its normal energy state which leads to the alteration and degradation of these substrates. Most PS is thought to act via Type II mechanisms, which ends with the production of singlet oxygen. The lifetime of singlet O_2 is very short (around 40 ns) and the radius of action is about 20 nm [24, 25].

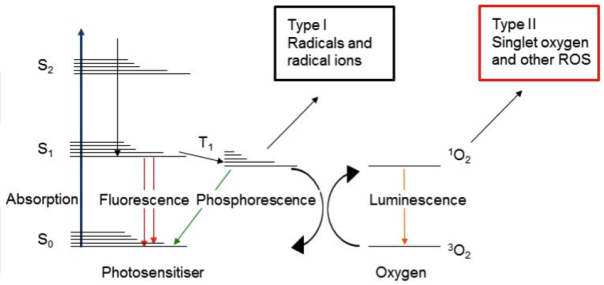

Figure 2.4 Schematic representation of Type I and Type II reactions following PS activation by irradiation [26].

2.4.2 Types of Photosensitizers

First of all, an effective PS should have high absorption coefficient and a triplet state of appropriate energy. As PS stays relatively long in triplet state more energy and/or electron transfer will occur. Therefore a highly effective PS has a high quantum yield of the triplet state and long triplet state lifetimes. Moreover, they have to have good cell penetration and have no dark toxicity.

Dyes such as rose bengal, eosin and methylene blue are very effective photosensitizers. Most photosensitizers currently used are derivatives of porphyrins, which are large aromatic rings containing nitrogen. They have the ability to absorb in the UV-VIS range.

2.4.3 Methylene Blue

Methylene blue (MB) is a widely known histological dye that has been in use for many years (Figure 2.5). It is a phenothiazinium dye with a strong absorbance in the range of 550-700 nm. MB localized towards the cytosol, lysosomes, mitochondria and cell nucleus. MB is well known for producing singlet O_2 in solution and in cellular environment [27].

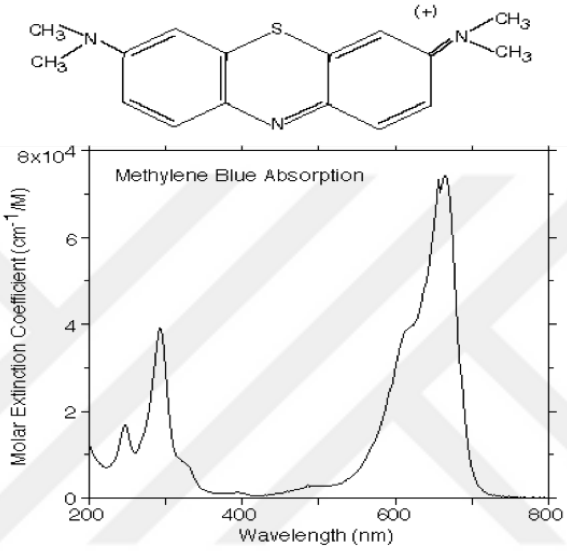

Figure 2.5 Methylene blue absorption spectrum and molecular structure [27].

2.4.4 Indocyanine Green

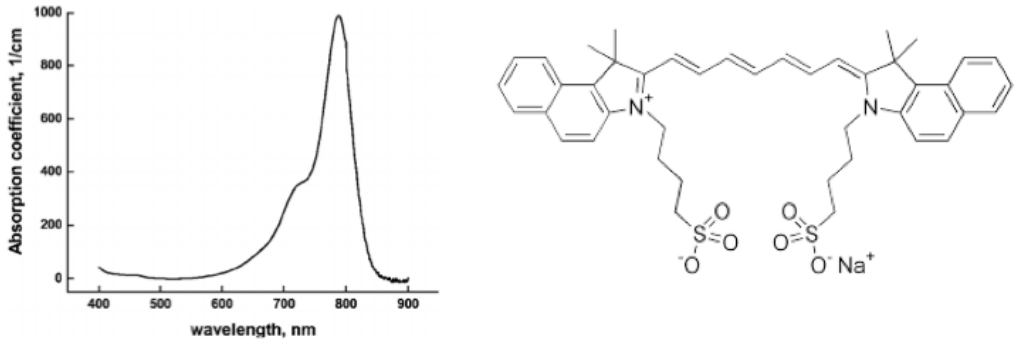

Figure 2.6 Indocyanine green absorption spectrum and molecular structure [28].

Indocyanine green (ICG) is a cyanine dye, which operates in near infrared region. It was approved by United States Food and Drug Administration (FDA) to be used

for medical procedures such as cardiac output imaging or retinal angiography [29]. The absorption spectrum depends largely on the solvent used and the concentration. ICG absorbs mainly between 600 nm and 900 nm. It has been proven that photoactivation of ICG in aqueous solution by irradiation generates singlet O_2 [30].

3. MATERIALS AND METHODS

The research is divided in 8 separate studies as given in Table 3.1. In each study several assays were performed. The Table 3.2 summarizes the experimental assays that were performed for both wavelengths and each protocol is explained in the following sections.

Table 3.1
Summary of the studies

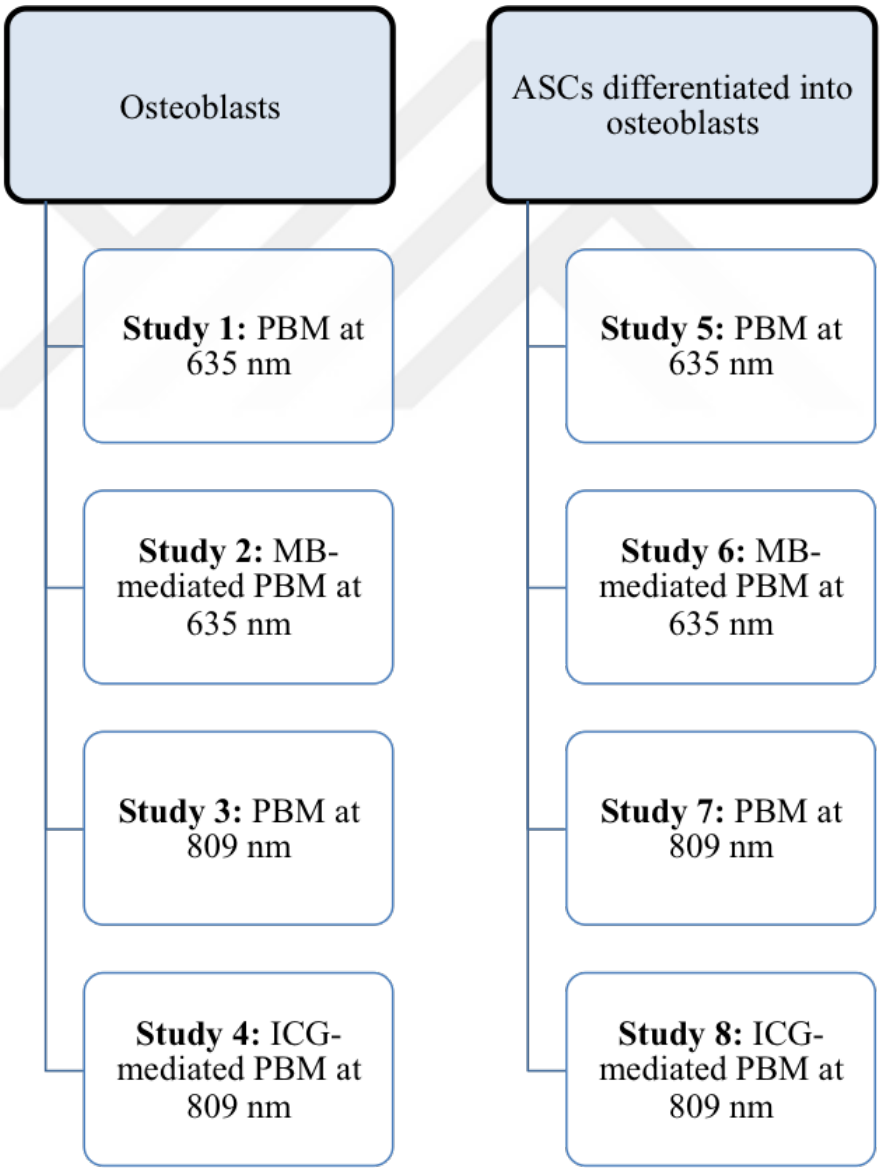

Table 3.2
Experimental assays performed in the research.

		Osteoblasts	ASCs
Cell Viability	MTT assay	at 24,48,72h	at day 7 and 14
Cell Viability	Acridine Orange/Propidium Iodide staining	at 24,48,72h	at day 7 and 14
Proliferation	Alamar Blue assay	at day 7 and 14	at day 7 and 14
ALP activity	ALP kit	at day 7 and 14	at day 7 and 14
Mineralization	Alizarin Red Staining	at day 7 and 14	at day 7 and 14
Gene expression	RT-PCR analysis	at day 7 and 14	at day 7 and 14
Singlet oxygen generation	DPBF solution	after laser irradiation	after laser irradiation
ROS presence	ROS fluorescent staining	after laser irradiation	after laser irradiation

3.1 Cell Culture

Human osteoblasts (ATCC® CRL-11372, Rockville, MD, USA) were grown at 37°C in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (Gibco, Grand Island, USA) without phenol red, supplemented by 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) and in humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 3 or 4 days.

The human ASCs used in this research was purchased (Invitrogen, CA, USA) and were grown at 37°C in low glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA), supplemented by 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) in humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 3 or 4 days. The cells were passaged when they reached 90% confluence with 0.25% trypsin-EDTA solution (Sigma, St. Louis, MO, USA). Osteogenesis was induced using culture medium supplemented with 1nM dexamethasone, 2mM β-glycerolphosphate and 50 μM ascorbate-2-phosphate. This osteogenic medium (ODM) were added to plates immediately after irradiation. The cells used in the experiments were at 2-4 passages.

3.2 Methylene Blue Application

Methylene Blue (Sigma, St. Louis, MO, USA) was dissolved in sterile PBS and filtered for each concentration during the experiments. The desired concentration of MB was added to the wells and the cells were incubated for 1 h at 37°C. After 1 h, the MB solution was replaced by PBS in order to prevent medium serum to interact with light and irradiation was performed for laser groups. Control and MB groups were kept outside the incubator for the same period of time as laser groups. The plates were protected from day light during all procedure.

For dosimetry experiments, concentration between 0.05-5.00 μM was used in order to find out a non-toxic concentration for osteoblasts. 0.05 μM concentration was chosen for the rest of the study.

3.3 Indocyanine Green Application

ICG ($C_{43}H_{47}N_2NaO_6S_2$) was purchased from Sigma (St. Louis, MO, USA). The green powder (1.0 mg/ml) ICG was dissolved sterile PBS to obtain stock solution. It was kept in dark and was diluted to the desired concentrations before performing the experiments. The cells were incubated for 1h at 37°C with desired concentration of ICG. Prior to laser application, ICG solution was replaced by PBS in order to prevent medium serum to interact with light. Concentrations ranging from 0.5 to 20.0 μM were applied to the osteoblasts to determine a non-toxic concentration for light coupled experiments. 0.5 μM concentration was chosen for the rest of the study. The plates were protected from day light during all procedure.

3.4 Laser Irradiation

Laser irradiation was carried out with two different diode lasers with 635 (visible red) and 809 nm (near infrared) (Figure 3.1). The in-lab developed computer controlled 809 nm diode laser system operated at a resulting power density of 50 mW/cm^2 at the level of cell surface which was measured using an optical power meter (1918-R, Newport Corp., CA, USA). For visible red illumination, a continuous diode red laser (635 nm-VA-I- 400-635, Optotronics-USA) with a maximum output power of 400mW was coupled to 600 um fiber. A collimator was used to have a homogeneous beam at the target area.

Table 3.3
Laser parameters

Laser Parameters		
Wavelength (nm)	635	809
Wave emission	Continuous	Continuous
Power density (mW/cm^2)	50	50
Energy density (J/cm^2)	0.5, 1 and 2	0.5, 1 and 2
Irradiation duration (sec)	10, 20 and 40	10, 20 and 40

Both laser setups were positioned to ensure that wells were irradiated homogeneously at a time from above. Blackout foil (Thorlabs Inc., NJ, USA) with an appropriate-sized hole was utilized to cover and protect the remaining wells from illumination and the lids of the plates were not off in order to reduce the risk of contamination. The amount of power density reaching the cell layer was reduced because of the plate lid [31]. This

power loss was considered and the position of the laser was set accordingly.

Three different energy doses were used: 0.5 J/cm^2, 1 J/cm^2 and 2 J/cm^2 which last 10, 20 and 40 s respectively for both wavelengths (Table 3.3). Those energy doses were chosen based on literature research [32–34]. Control cells were maintained outside the incubator under the same condition as irradiated cells. The medium was changed with PBS before laser treatment in order to prevent medium serum to interact with light. All of the experiments performed in triplicate. For ASCs, ODM was added to all groups immediately after irradiation and it was changed every 3 days. The cells were collected at various times for analysis according to the purposes of the experimental protocols.

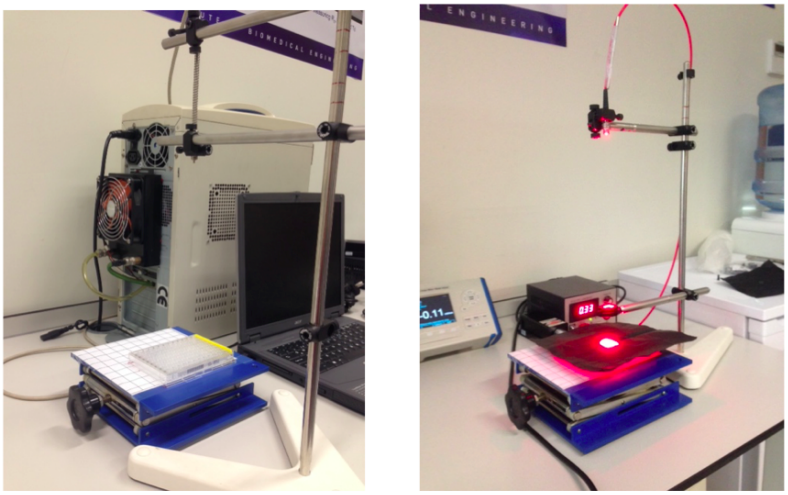

Figure 3.1 Laser systems used in the experiments.

3.5 Cell Viability: MTT Assay

Morphological conditions of cells were observed daily with an inverted microscope. Cell viability was evaluated by -[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay at 24, 48 and 72 h post irradiation or at 7^{th} and 14^{th} day. MTT is a standard colorimetric assay in which the reduction of yellow MTT to purple formazan was measured spectrophotometrically [35, 36]. The cells were seeded in 96-well plates at a cell density of 1x10^5 cells/well. After 24 h incubation for adhesion, they were either irradiated or used as control group. 10 μl of MTT solution (5 mg/ml) was added to each well containing 100 μl of medium and the plates were incubated

for 4 h at 37°C. Then the reaction was terminated by removing the MTT solution and adding 150 μl DMSO. The plates were folded in aluminum foil and agitated on a plate shaker for 15 min. The optical absorbance was read at 570 nm on the microplate reader (iMark, Bio-Rad). To obtain net absorbance values, average absorbance values of blank wells were subtracted from values of sample wells and then the values were normalized.

3.6 Cell Proliferation: Alamar Blue Assay

Alamar Blue (AB) is a water-soluble, non-toxic dye, which is used to quantify cell proliferation [37]. The cells were seeded in 96-well plates at a cell density of 1x105 cells/well, incubated for 24 h prior to laser treatment. At 24, 48 and 72 h post irradiation or at 7^{th} and 14^{th} day, 10% AB (Invitrogen, CA, USA) was added to each well and the plates were incubated for 4 hours at 37°C. The optical absorbance was read at 570 nm. The relative cell proliferation was normalized to the untreated control.

3.7 Acridine Orange/Propidium Iodide Staining

Acridine Orange (AO) and Propidium Iodide (PI) are dyes that bind to nucleic acids and can be used to measure the cell viability. AO is dye that can pass through cell membrane so the viable cells nuclei are stained by AO. However, PI, which is membrane impermeable, can only enter the cells that their membrane integrity is disrupted. Viable cells show green fluorescence, non-intact dead nucleated cells fluoresce red [38].

Cells were seeded in 96 well tissue culture plates ($1x10^5$ cells/well). After 24 h incubation, they were irradiated or not. The staining was performed at 24, 48 and 72 h (or 7^{th} and 14^{th} day for ASCs) after irradiation. The cells were stained with mixture of AO (2 μg/ml- Sigma,St. Louis, MO, USA) and PI (2 μg/ml- Sigma, St. Louis, MO, USA) and observed immediately using an inverted microscope with a fluorescent attachment (Leica dfc295). Multiple independent fluorescent micrographs were taken. The ratio between

viable to dead cells was measured using ImageJ program (National Institute of Health, USA). The color image (RGB), as in the Figure 3.2 above, is converted to greyscale image first. Finally a binary image is created and cells were counted by the program.

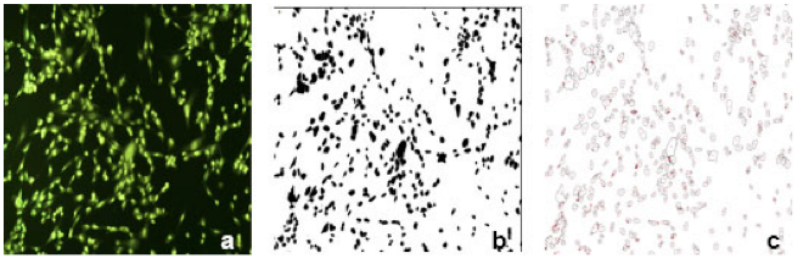

Figure 3.2 The cells were counted by the ImageJ program. a) color micrograph, b) greyscale image, c) binary image created by the software.

3.8 Alizarin Red Staining for Mineralization Evaluation

Bone mineralization was visualized with Alizarin Red staining at days 7 and 14. Attached cells were washed with PBS and fixed in 10% formalin for 30 minutes at room temperature. Then the formalin was removed and the cells were washed twice with deionized H_2O. 2% Alizarin Red S (Sigma, St. Louis, MO, USA) solution, pH 4.2, was used to stain the cells for 10 minutes. Excess solution was removed and the cells were washed with PBS again. Calcification deposits, which were bright red/orange under an optical microscopy, were photographed. The red matrix precipitate was solubilized in 15% acetic acid, 20% methanol solution for 45 min in dark and the optical density of the solution was read at 405 nm [39].

3.9 Alkaline Phosphatase Activity

Alkaline Phosphatase (ALP) activity was assayed using a commercial colorimetric kit (Biovision Research Products, CA, USA) at 7 and 14 days. Briefly, the samples were added to 96-well plates and the volume was brought to 80 μl and 50 μl of 5mM p-nitrophenyl phosphate solution was added to each well, leading to the formation of a

yellow p-nitrophenyl. After 60 min incubation in dark at room temperature, 20 μl of stop solution was added and absorbance was measured at 405 nm.

3.10 Fluorescence Staining of Reactive Oxygen Species

A ROS detection kit (Enzo Life Sciences Inc, Farmingdale, NY) is used to directly monitor real time reactive oxygen and/or nitrogen species (ROS/RNS) production in live cells using fluorescence microscopy. All procedures were performed in accordance with the manufacturer's protocol. Basically, the kit uses a cell-permeable oxidative stress detection reagent, which will emit green fluorescence when it directly reacts with different ROS types. Aliquots of $1x10^5$ cells in 100μl of culture medium were seeded in 96-well plates and allowed to adhere overnight. Next day, the cells were treated with ROS Detection Solution prepared according to the kit's manual for 1 h. Positive and negative control are also established by treatment with ROS Inducer and ROS inhibitor. Before laser irradiation (previously incubated with PS for PS-mediated PBM groups), ROS solution was replaced with PBS. Finally, cells were washed with wash buffer and observed under fluorescence microscope.

3.11 Singlet Oxygen Generation

The singlet oxygen generation give an indication of the efficiency PS [40]. The singlet oxygen generation was determined using a chemical method (1,3-diphenylisobenzofuran (DPBF) in ethanol. The disappearance of DPBF was monitored using a UV-Vis spectrophotometer. Singlet oxygen causes degradation of DPBF [41]. DPBF reacts irreversibly with 1O2 that causes a decrease in the intensity of the DPBF absorption band at 400 nm [42]. The DPBF solution including 0.5 μM ICG or 0.05 μM MB were irradiated with 809 and 635 nm laser light respectively for each energy densities of 0.5 J/cm^2, 1 J/cm^2 and 2 J/cm^2. The absorption spectrum was recorded using a UV-Vis spectrophotometer.

3.12 Real Time Polymerase Chain Reaction (RT-PCR) Analysis

To better characterize cellular function, relative gene expression levels between the control and irradiation groups were evaluated by RT-PCR. Total RNA was isolated using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and cDNA synthesis was performed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The markers for osteoblast phenotype were COL1A (collagen type I), ALPL (alkaline phosphatase) and BGLAP (osteocalcin). All samples normalized to total RNA content. The housekeeping gene that is used for normalization was GAPDH. Fold differences in gene expression were calculated using the 2 deltadelta Ct method ($2^{\Delta\Delta Ct}$) [43] . The LigthCycler 480 with multiwell plates 96 program was set at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s followed by 30s of cooling at 40°C.

3.13 Statistical Analysis

One-way analysis of variance (ANOVA) supplemented with Tukey's test or Kruskal-Wallis complemented by Mann-Whitney tests are performed for statistical comparison by using Statistical Package for the Social Sciences (SPSS) v 22.0 software (SPSS Inc., Chicago, IL). The differences between experimental groups are considered to be statistically significant at $p < 0.05$.

4. RESULTS AND DISCUSSIONS

4.1 STUDY 1: The effects of photobiomodulation (635nm) on human osteoblasts

4.1.1 Aim

The aim of this study was to investigate the effect of red light at 635 nm on human osteoblasts in a well-designed manner. Cell viability, proliferation, ALP activity, mineralization and specific gene expressions were analyzed.

4.1.2 Results

Cell viability was assessed by MTT assay. The normalized absorbance after 635 nm laser application are given in the Figure 4.1. Although there was a slight increase in cell viability 48 h after irradiation in the 2 J/cm^2 group compared to the control, the effect was not statistically significant. No differences in cell viability were found at 24 h or 72 h post irradiation for the same wavelength.

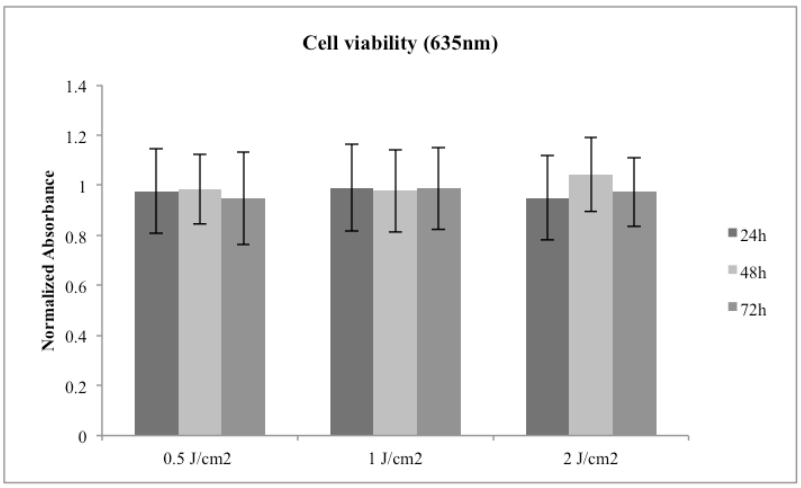

Figure 4.1 The normalized absorbance (570 nm) of Alamar Blue assay at 24, 48 and 72 h. No significant differences were observed between groups ($p>0.05$).

Table 4.1
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ No significant differences were observed between groups ($p>0.05$).

635 nm	**24 h**	**48 h**	**72 h**
0.5 J/cm²	99.0% ± 1.2	102.0%± 1.3	97.0% ± 0.8
1 J/cm²	101.0% ± 2.5	100.0% ± 0.9	96.0% ± 0.4
2 J/cm²	99.0% ± 1.9	98.0% ± 1.1	99.0% ± 2.7

The Alamar Blue test was performed in order to examine cell proliferation for the first 72 h after irradiation. No significant changes in proliferation were observed after laser application. The normalized cell proliferation percentages are shown in the Figure 4.2.

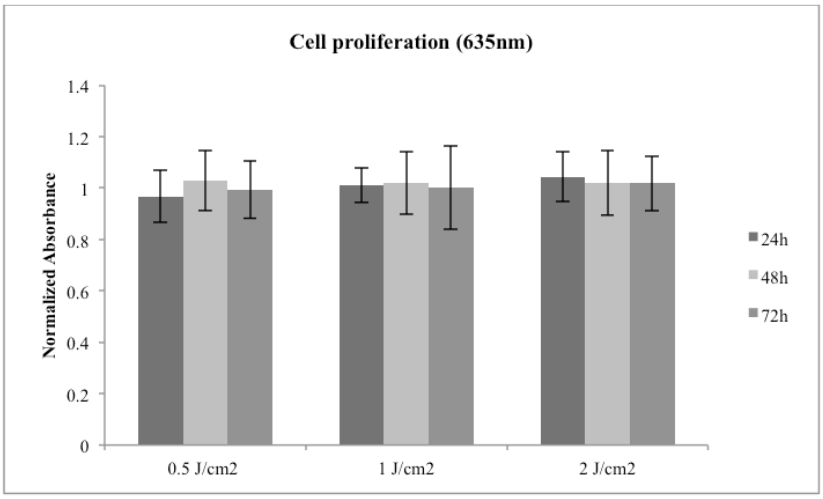

Figure 4.2 Normalized absorbance at 570 nm for MTT assay. No statistical differences were observed ($p> 0.05$).

To visualize cell viability, AO/PI staining was performed. AO is a cell permeable dye, green fluorescence is emitted by live and intact cells. PI, which enters the cells whose membrane integrity is disturbed, will cause reddish fluorescence (Figure 4.3). Fluorescent micrographs are taken and live/dead cells are counted by using ImageJ analyses program. The mean percentage changes in osteoblast viability were given in the Table 4.1. No differences were observed between laser groups and control cells.

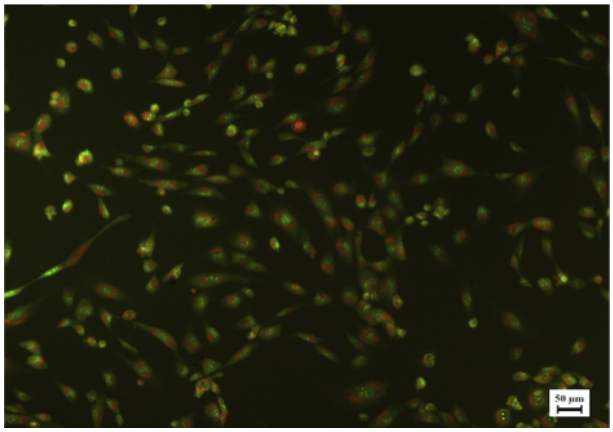

Figure 4.3 An example of micrograph after AO/PI staining. Scale bar: 50 μm.

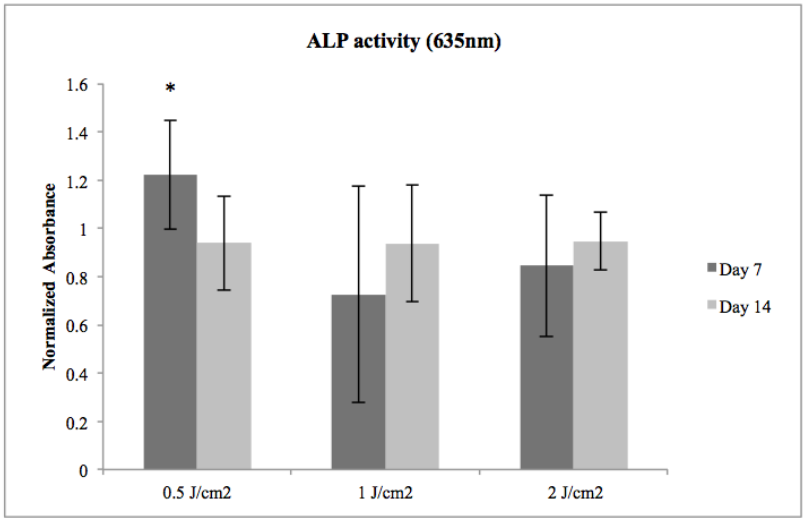

Figure 4.4 Normalized absorbance values for ALP activity assay. At day 7, 0.5 J/cm^2 laser irradiation caused significant increase in ALP activity of osteoblasts compared to the control group. * indicates statistically significant to the control group ($p<0.05$).

At day 7, 0.5 J/cm^2 laser irradiation caused significant increase ($p<0.05$) in ALP activity of osteoblasts compared to the control group (Figure 4.4). ALP activity in osteoblast cells was not altered by 635 nm laser irradiation at 14^{th} day.

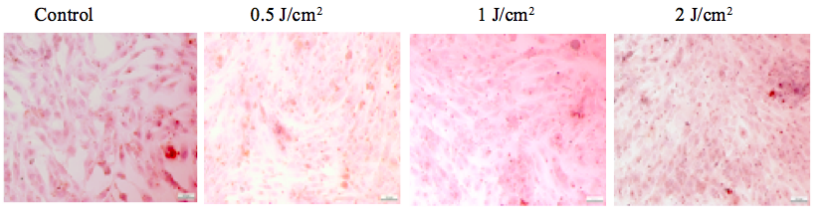

Figure 4.5 Microscopic images of osteoblasts (10x) treated with different doses of laser irradiation at day 14 with Alizarin red. Scale bar:100μm. The orange dots inside the cells are calcium nodules that are stained by the dye.

Alizarin red staining was performed in order to see bone mineralization nodules at 7^{th} and 14^{th} day. All groups showed mineral deposits as seen in the micrographs (Figure 4.5). The orange dots inside the cells are calcium nodules that are stained by the dye (Figure 4.6) shows the normalized absorbances at 405 nm in order to quantify Alizarin red staining. Except 1 J/cm^2 group at day 14, all the groups at both time periods had significantly decreased mineralization compared to the control group ($p<0.05$).

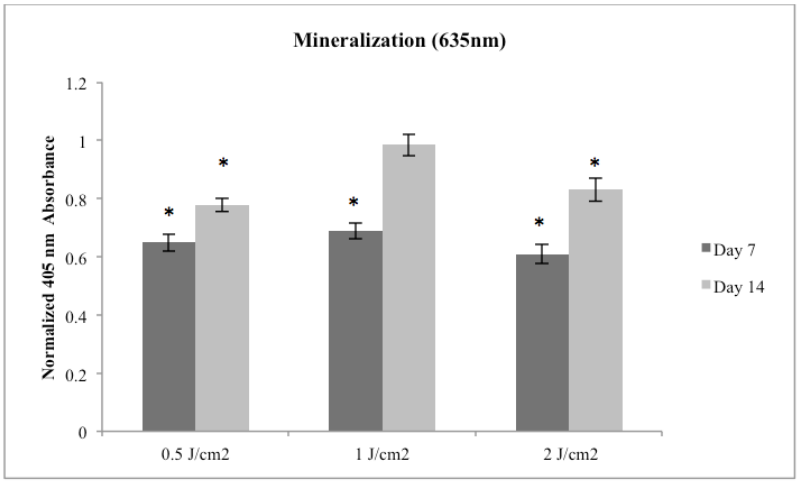

Figure 4.6 Normalized absorbance at 405 nm after Alizarin Red staining .The mineralization was decreased significantly after laser irradiation for all groups except 1 J/cm^2 group at day 14.* indicates statistically significant to the control group ($p<0.05$).

A statistically significant down regulation of BGLAP was observed with a laser dose of 0.5 J/cm^2 and to a lesser extent in 1 and 2 J/cm^2 irradiations for both time periods. No evident fold-increase was detected for ALPL gene between laser irradiated/non-irradiated cells except for 1 J/cm^2 group at day 7, which was significantly decreased. 635 nm laser exposure at 2 J/cm^2 dose caused up regulation of COL1A gene. On the other hand, the relative expression was increased for 2 J/cm^2.

4.1.3 Discussion

The results demonstrate that overall there were no differences on cell viability and proliferation between control and experimental groups after a single laser irradiation at 635 nm wavelength according to the MTT, Alamar Blue and AO/PI staining results. The energy doses used in this study were chosen similar to those in several previous studies

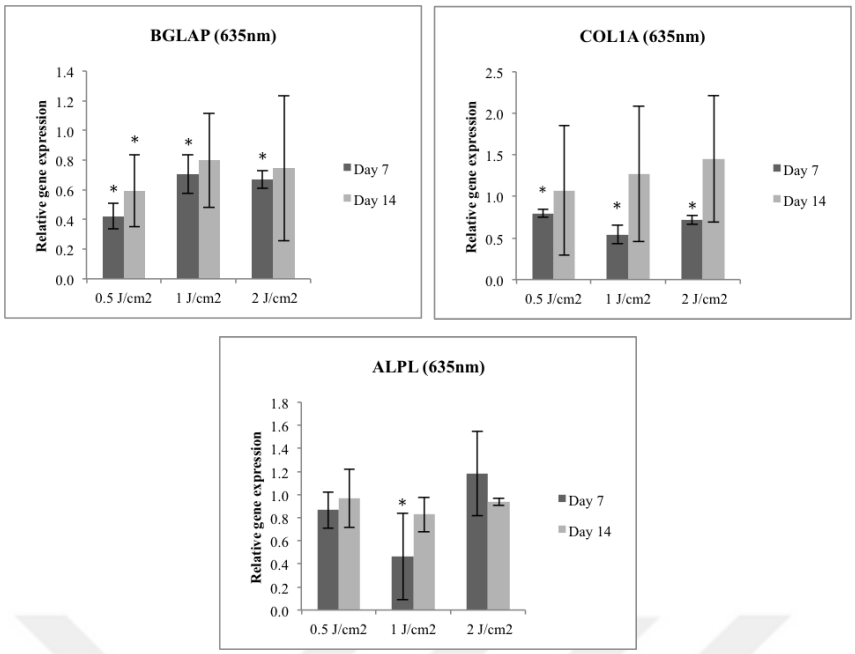

Figure 4.7 The relative gene expressions normalized to GAPDH at 7^{th} and 14^{th} day. * indicates statistically significant to the control group ($p<0.05$).

that investigated PBM. There exist controversial results between studies investigating the effect of red light on proliferation of osteoblasts or osteoblast-like cells.

Similar to results of the present study, Renno et al. did not observe any statistically significant change in cell viability and proliferation after 670 nm laser irradiation of normal primary osteoblasts (MC3T3) [44]. In another research, SAOS-2 cells were irradiated with 1 and 2 J/cm^2 using a diode laser with 670 nm. Cell viability test at 24, 48 and 72 h did not show significant difference between laser and control groups [45]. Another research group tested the effect of higher energy densities at 685 mm (25, 77, and 130 J/cm^2) on osteogenic cells originated from rat calvaria. They concluded that those parameters did not influence cell growth and proliferation [46].

In contrast, some studies have demonstrated a significant increase in cell viability and proliferation after PBM. Pagin et al. found that at 24 h of irradiation, cell growth of MC3T3 cells was stimulated by 660 nm laser at 3 and 5 J/cm^2 and LED at 5 J/cm^2 [47]. In another study, human osteoblast cells derived from bone were irradiated by a 632 nm laser on days 2 and 3 after seeding (0.14, 0.43, and 1.43 J/cm^2 energy densities). According to their MTT results, an increase of 40.0% and 38.0% in optical density (OD)

was observed 24 and 48 h after the second irradiation, respectively [48]. In another study, human osteoblast-like cell line (SAOS-2) was exposed to laser irradiation at 1 or 3 J/cm^2 energy densities with a semi-conductor laser diode (659 nm). Significantly increased cell proliferation were detected between dark control and irradiated groups on day 2 post irradiation. No differences were found on days 3 and 7. The authors hypothesized that a single dose effect may not last even if it may exert a biostimulative effect in the first few days [49].

ALP activity is another critical behavior of osteoblasts. Its concentration is high at beginning but decreases as the matrix becomes more calcified. Except 0.5 J/cm^2 group at day 7 ($p<0.05$), no significant changes have been observed in ALP activity of the cells. Moreover, although it is not significant, a decreasing trend has been noted at day 7 in 1 and 2 J/cm^2 groups. The ALPL gene expression is also down regulated for 1 J/cm^2 group at day 7. However, no significant changes have been observed in other groups or time period. Similar to the present study, ALP activity was not altered after red light irradiation in other studies [47]. In contrast, a two-fold enhancement of ALP activity in the irradiated cells as compared to non-irradiated osteoblasts has been measured in the study of Stein at al [48]. In another study, using a diode laser with 670 nm at an energy density of 2 J/cm^2 resulted in persistently lower alkaline phosphatase activity in Saos-2 cells [45].

Although cell viability and ALP activity are not evidently affected by 635nm irradiation, mineralization and gene expressions have been altered. When both cell viability and Alizarin red staining results are examined, it seems that the significant decrease in the amount of calcium depositions following 635 nm light irradiation was not attributable to the decrease in cell number, but rather to a decrease in mineralization level of the existing cells. Except 1 J/cm^2 group at day 14, all the groups at both time periods had significantly decreased mineralization compared to the control group ($p<0.05$). BGALP expression, which is a late period marker in matrix maturation, was significantly decreased after irradiation in all groups at day 7 and for 0.5 J/cm^2 group at day 14. Mature osteoblasts can form extracellular matrix. Type I collagen synthesis and collagen accumulation do not occur at the same time in developing osteoblasts. First collagen synthesis peaks, then

matrix accumulates when collagen synthesis is decreasing [50] The RT-PCR data of this study revealed that COL1A seem to be the mostly expressed one compared to BGALP or ALPL at 14^{th} day. Therefore, mineralization may not be completed yet. The decreased level of BGALP expression may be considered another sign of incomplete mineralization, which is consistent with decreased calcium staining results.

Taking together our results and other investigations, it can be stated that PBM at the studied energy doses at 635 nm did not affect osteoblast viability and proliferation. Similarly, laser irradiation did not appear to have any meaningful effect on ALP activity. These results were confirmed by RT-PCR analysis of osteoblast markers. However, the relationship between collagen synthesis and mineralization remains unknown necessitating further study.

4.2 STUDY 2: Methylene Blue mediated photobiomodulation (635nm) on human osteoblasts

4.2.1 Aim

In the present study, the aim was to investigate the effect of 635 nm laser light at three different energy densities (0.5, 1 and 2 J/cm^2) mediated by MB on the osteoblast behavior. The interaction of light with MB may have a synergetic effect that may lead to cell proliferation or the combined effect may still cause cell death. The cell viability and proliferation was measured quantitatively (MTT and Alamar Blue assays) and qualitatively (AO/PI staining). The ALP activity, mineralization and gene expressions of phenotypic osteoblast markers (ALPL, BGLAP, COL1A) were also assessed to better investigate the possible effects of MB mediated PBM. The increase in the amount of ROS is tested by ROS staining and singlet oxygen measurement was performed by DPBF solution.

4.2.2 Results

The first step was to determine the non-toxic dose of MB, which will be combined with laser irradiation. For dosimetry experiments, concentration between 0.05-5 μM was used and 0.05μM concentration was chosen for MB-mediated PBM experiments. According to the MTT results, among the concentration between 0.05 and 5 μM, the concentrations higher than 0.5 μM may be considered as toxic, being LD_50, at the end of the 72h ($p<0.05$). For the toxic concentrations, viability decreased more following each 24h. The doses 0.05, 0.1 and 0.25 μM did not cause any significant change in cell viability percentage (Figure 4.8). 0.05 μM was chosen for laser experiments in order to deliver less photosensitizer to the cells.

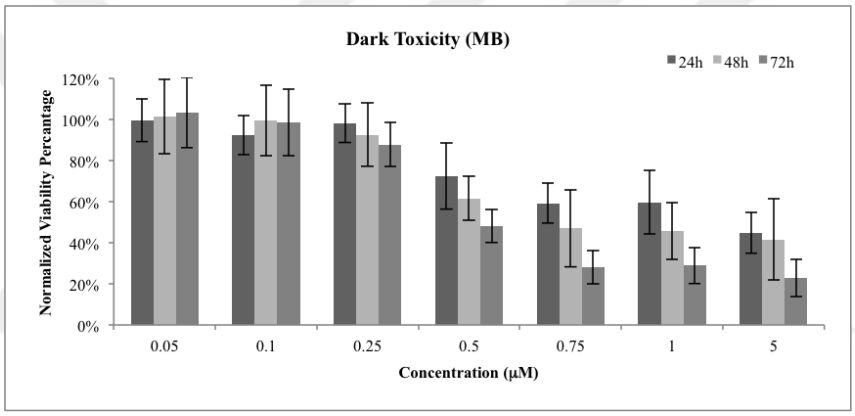

Figure 4.8 Cell viability of osteoblasts treated with different concentration of methylene blue. The concentration higher than 0.5μM showed more than 50% cell death ($p<0.05$) compared to the control.

When light is applied after MB incubation, it seems that there is a decreasing trend in cell viability with respect to time. The decrease is statistically significant at 72h and there are not any differences between different energy densities (Figure 4.9). According to Alamar Blue assay results, no significant differences in proliferation of osteoblasts were observed in the different laser applications when compared to the control and MB group (Figure 4.10).

AO/PI staining was performed after 24,48 and 72 h of MB and laser application. On fluorescent micrographs, live cells have a normal green nucleus whereas necrotic cells are detected by their red/orange nuclei (Figure 4.11). Combined MB and laser groups seem to have more dead cells in micrographs. However, when the ImageJ program has

counted the cells on the micrographs, no statistical difference was found.

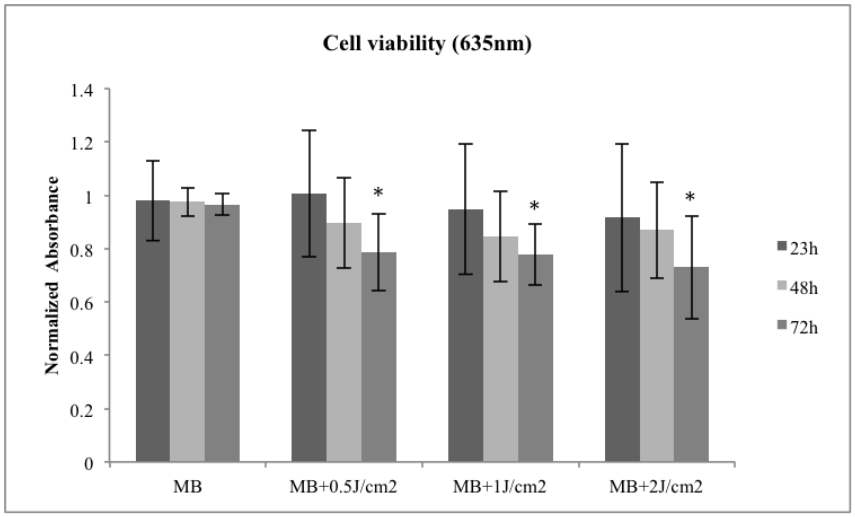

Figure 4.9 Normalized absorbance at 570 nm for MTT assay performed 24,48 and 72 h after treatment. MB concentration was 0.05 μM. * indicates statistically significant difference ($p<0.05$) compared to the control group.

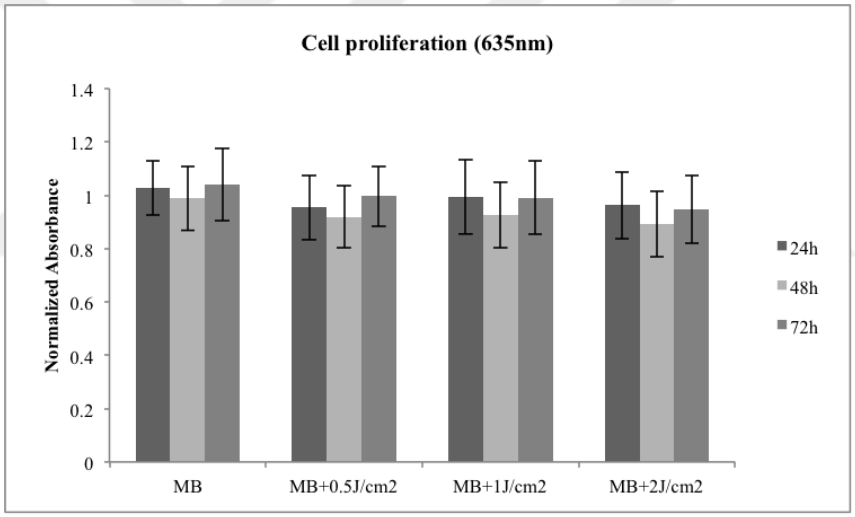

Figure 4.10 The normalized absorbance (570 nm) of Alamar Blue assay at 24, 48 and 72 h. MB concentration was 0.05μM. No significant differences were observed between groups ($p>0.05$).

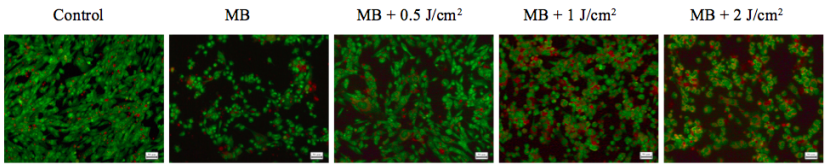

Figure 4.11 Fluorescent micrographs of AO/PI stained osteoblasts at 72 h after treatment (Scale bar:100 μm). Green fluorescence show live cells, red fluorescence show dead cells. Dead cells seem to be more populated for MB+laser groups. MB concentration was 0.05μM.

Table 4.2
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ.. No significant differences were observed between groups ($p>0.05$).

635 nm	**24 h**	**48 h**	**72 h**
MB	96.0% ± 2.2	99.0% ± 0.3	98.0% ± 4.6
MB+ 0.5J/cm^2	99.0 %± 1.6	96.0% ± 2.1	95.0% ± 1.3
MB+ 1J/cm^2	93.0% ± 3.4	98.0% ± 1.8	99.0% ± 0.4
MB+ 2J/cm^2	96.0% ± 3.2	97.0% ± 1.8	95.0% ± 2.1

The ALP activity, which is crucial for initiation of mineralization, was shown in Figure 4.12. Application of MB did not cause any change in ALP activity but when light is combined to MB treatment, it was increased significantly for the three energy densities at 7^{th} day ($p<0.05$). However, this effect was not observed for the 14^{th} day and the results were similar in control and treatment groups.

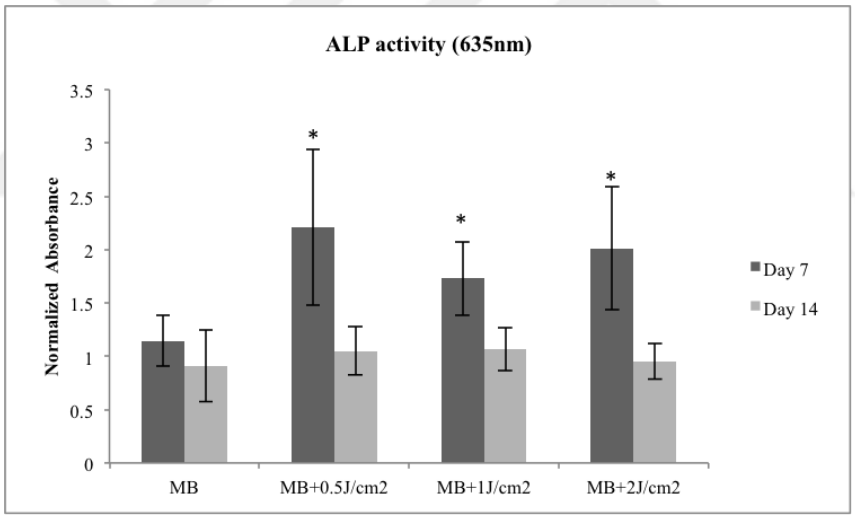

Figure 4.12 Normalized ALP activity measured at 7^{th} and 14^{th} day. All MB-laser groups have significantly increased ALP activity at day 7. * indicates statistically significant difference ($p<0.05$).

Osteoblasts were subjected to Alizarin Red staining for calcium content at 7^{th} and 14^{th} day after treatments. Mineralized nodules were detected on the micrographs of all groups for both time points. Quantitative analysis of calcium deposits showed that mineralization is lower at 7^{th} day compared to 14^{th} day for all groups as expected ($p<0.05$). However, for both days MB and laser combined groups showed significantly less mineralization compared to control group (Figure 4.14).

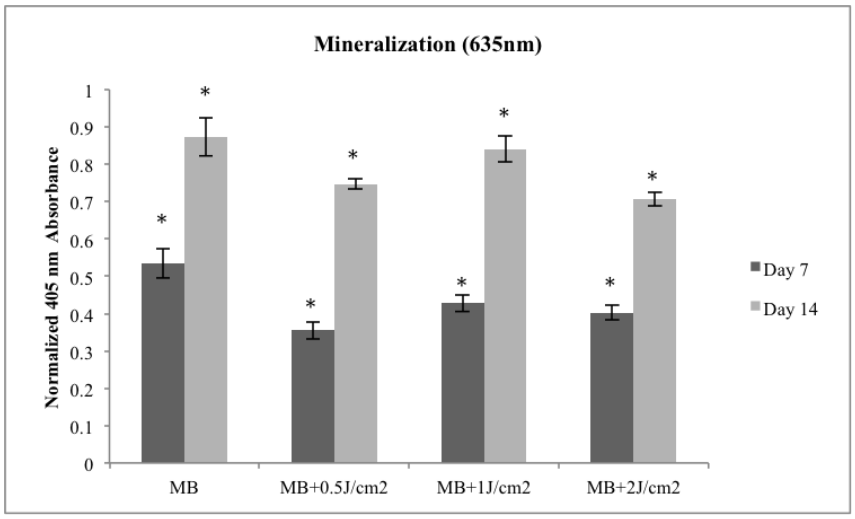

Figure 4.13 Normalized absorbance at 405 nm after Alizarin Red staining. MB concentration was 0.05 μM. All of the treatment groups had significantly lower mineralization compared to the control group. * indicates statistically significant difference ($p<0.05$) compared to the control group.

The expression of BGALP ALPL and Col1A were analyzed by RT-PCR at 7^{th} and 14^{th} day (Figure 4.14). BGALP gene expression was almost the same at both time points even though there is a slight decrease, no significant difference between control and treatment groups was observed. The ALPL expression seems to be not affected by treatment except for MB+2 J/cm^2 group, which has a slight increase in the expression ($p<0.05$). At the 14^{th} day, the most expressed gene was COL1A compared to ALPL and BGALP. In the MB+2 J/cm^2 group its expression was significantly higher than the control group. ALPL expressions seem to be slightly but not significantly increased for all groups.

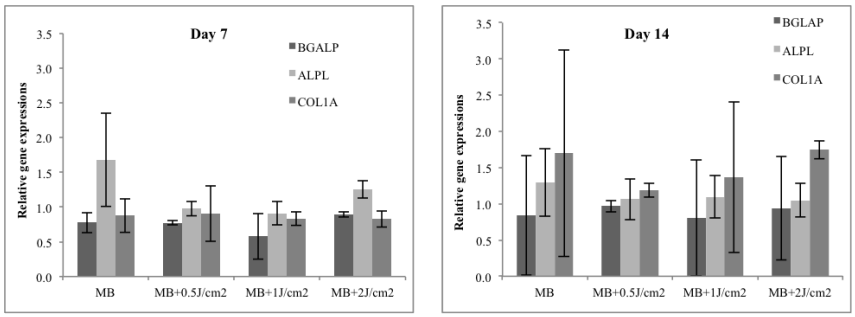

Figure 4.14 Relative gene expression at day 7 and 14. MB concentration was 0.05 μM. * indicates statistically significant difference ($p<0.05$).

Singlet oxygen generation was performed using the trap molecule 1,3-diphenylisobenzofuran (DPBF) in ethanol. Only MB did not cause any change in absorbance peak of DPBF. When the light is combined with MB, singlet oxygen production started and absorbance

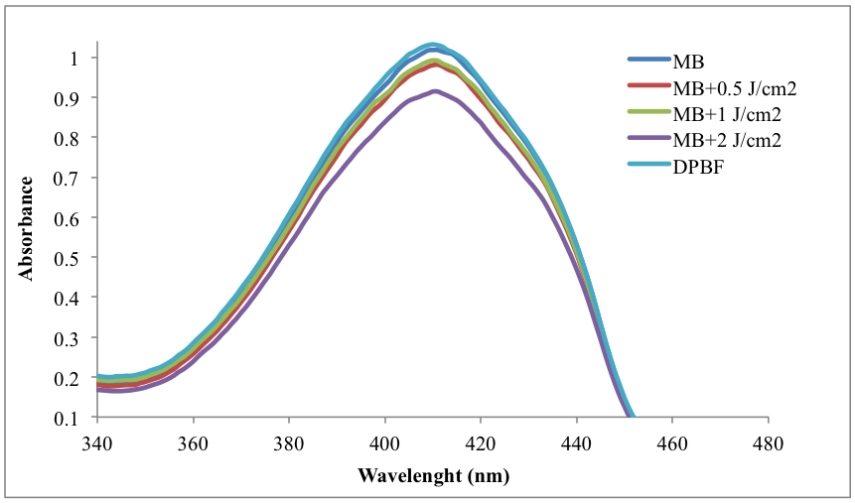

Figure 4.15 Decrease in absorbance spectrum of DPBF after MB and laser irradiation.

of DPBF, which peaks at 414 nm decreases. As energy density increases, the decrease in DPBF absorbance increases (Figure 4.15). ROS staining with detection kit have demonstrated that every sample group showed ROS presence. The micrographs are given in (Figure 4.16)

4.2.3 Discussion

The aim of the present study was to determine the effects of PBM mediated by Methylene Blue. MB is one the most effective phenothiazine PS *in vitro*, but has also been shown to be effective *in vivo* if it is administered intratumorally [51, 52]. In the present study, first its dark toxicity was investigated. Kirste et al. has found that LD50 concentration of MB on the RIF-1 cells was about 150 μM [53]. In our study, a range of concentration less than 100μM has been applied (not all data are shown). Concentrations higher than 0.5 μM have been found to be toxic for osteoblasts after 1h incubation. The lowest concentration that did not have any negative effect was chosen in order to deliver less PS to the cells and minimize possible side effects. The light combined studies were conducted using MB 0.05 μM concentration. The laser light that will be combined with MB was at 635 nm since MB has strong absorption in this region. The generation of singlet oxygen is shown by the decrease in the peak of DPBF molecule. ROS presence was detected by ROS staining kit in all groups.

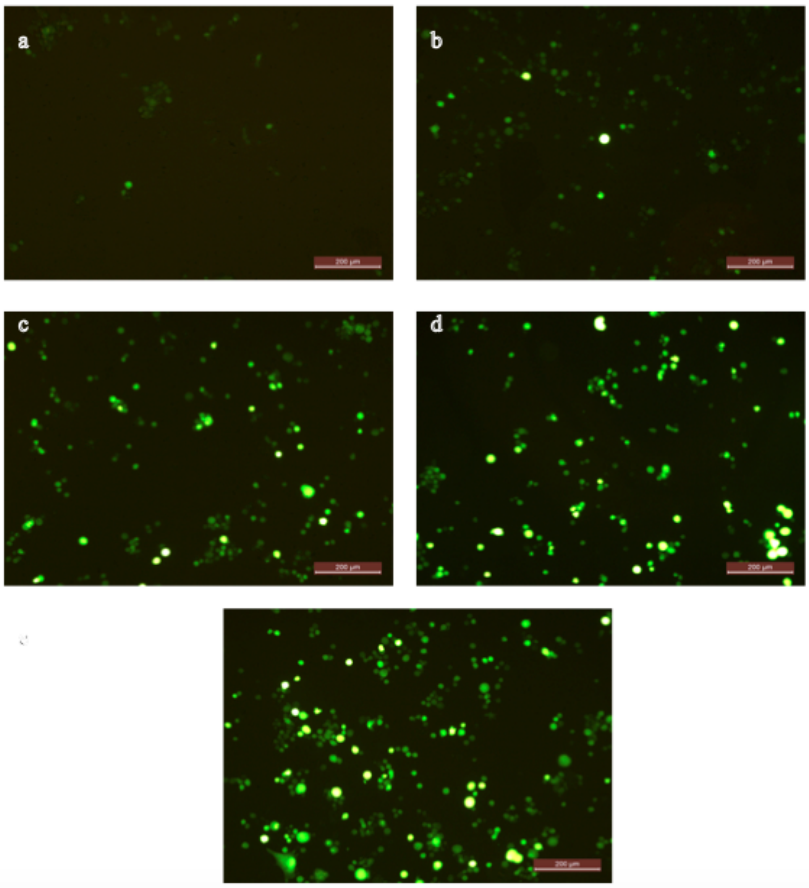

Figure 4.16 Micrographs showing ROS staining after MB and laser irradiation. scale bar:200 μm. a) control, b) MB, c) MB+0.5 J/cm^2, d) MB+1 J/cm^2 e) MB+2 J/cm^2

MTT assay has demonstrated that when laser light is combined with MB, there was a slight decrease in cell number, especially 72 h after the treatment. However, the decrease was about 20% and does not reach LD50 level. In the study I, the same energy densities (0.5,1 and 2 J/cm^2) were used to irradiate osteoblasts and no obvious effect on cell viability has been observed [54]. Cell death in MB-laser groups may also be observed qualitatively by AO/PI staining. On the other hand, cell proliferation determined with Alamar Blue assay does not seem to be affected by MB and red light application. Even though cell viability and proliferation have not been dramatically affected, more detailed analysis on mineralization, ALP activity and gene expressions revealed some differences. When mineralization level of treatment groups was compared to control group, there was dramatic decrease in the calcium depositions. The effect was more obvious at day 7 but it was still significant at day 14 even though the values approaches to the control group values. The slight decrease in BGALP expression assessed by RT-PCR may explain the

low level of mineralization since BGALP is a late period marker in matrix maturation [55]. The ALP activity of combined groups is significantly higher at day 7. The RT-PCR analysis agreed with this increased level of ALP in the MB-2 J/cm^2 group, showing increased ALPL expression. The ALP is a marker of osteoblast differentiation and its production is necessary for the initiation of mineralization [56]. At the 14^{th} day, the most expressed gene was COL1A. Mature osteoblasts can form extracellular matrix, which occurs when collagen synthesis decreases [50]. The significant increase in the COL1A expression of the MB-2 J/cm^2 group may be an indicator that mineralization is not completed yet. Moreover, some studies suggest that even though ALP activity increases, the collagen matrix cannot be mineralized because of the other chemical in the medium. Or even the level of ALP itself can decrease mineralization [57]. Therefore, although MB-combined PBM did not cause severe cell death, it may decrease osteoblast specific behavior.

There are few studies that investigate PS and light for biostimulative purposes. Chellini et al. used Nd-YAG laser (at 50 and 70 Hz) to irradiate Saos-2 cells with an energy density of 1.5 J/cm^2 after pre-treatment with MB (0.01% for 2 minutes incubation). They concluded that even if the cell viability was not affected by MB application itself, it might affect cell functions and reduce proliferation and differentiation. To sum up, MB-mediated 635 nm laser irradiation at indicated energy doses did not cause any biostimulative synergetic effect on osteoblast cells.

4.3 STUDY 3: The effects of photobiomodulation (809nm) on human osteoblasts

4.3.1 Aim

The aim of this study was to investigate the effect of infrared light at 809 nm on human osteoblasts. Cell viability, proliferation, ALP activity, mineralization and specific gene expressions were analyzed.

4.3.2 Results

The colorimetric MTT assay was used to assess cell viability. The normalized absorbance of MTT assay for 809 nm laser application are given in the Figure 4.17. Slightly increased cell viability was observed in all laser groups at the end of 48 h but the effect is not seen at 24 or 72 h.

The Alamar Blue assay was performed in order to examine cell proliferation for the first 72 h after irradiation. No significant changes in proliferation were observed after laser application. The normalized cell proliferation percentages were shown in the Figure 4.18.

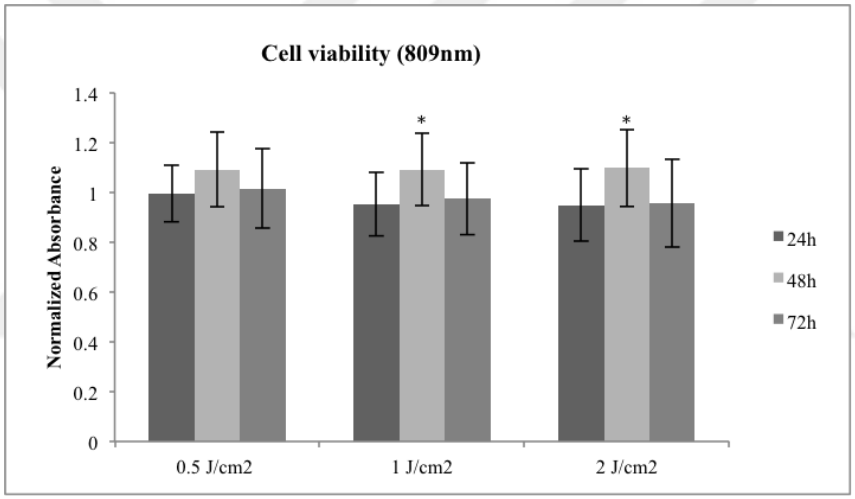

Figure 4.17 Normalized absorbance at 570 nm for MTT assay 24,48 and 72 h after laser irradiation. Significant increase in cell viability after 809 nm laser irradiation at 48 h ($p<0.05$) * indicates statistically significant difference ($p<0.05$) compared to the control group.

AO/PI staining was performed in order to visualize under fluorescent microscope and quantify live/dead cells by ImageJ software. The data are given in Table 4.3. No statistical difference was found when experimental groups are compared to control group ($p>0.05$).

Laser irradiation at 809 nm triggered a minor, but not statistically significant, increase in ALP activity at a dose of 1 J/cm^2. Other dose exposures did not cause any change in ALP activity (Figure 4.19).

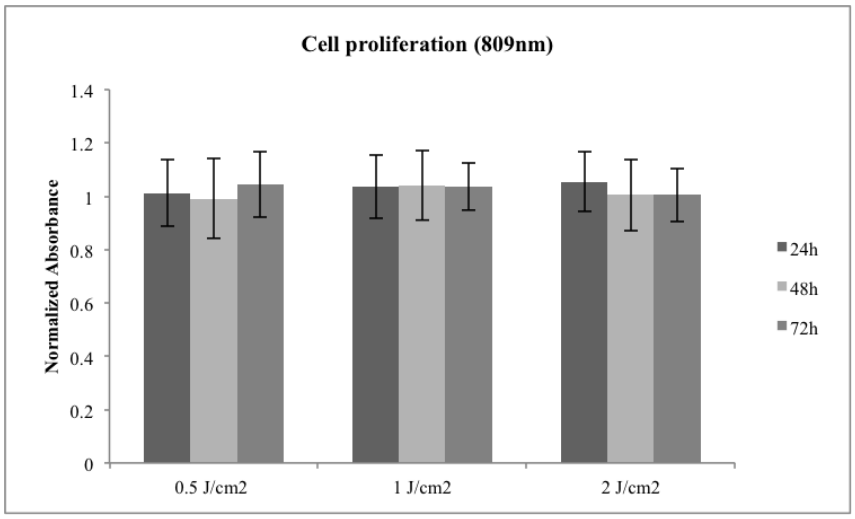

Figure 4.18 The normalized absorbance for Alamar Blue assay which measures proliferation at 24, 48 and 72 h. No significant differences were observed between groups ($p>0.05$).

Table 4.3
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ. No differences were observed between laser groups or control cells.

809 nm	**24 h**	**48 h**	**72 h**
0.5 J/cm²	101.0% ± 1.4	99.0 %± 0.9	97.0% ± 1.7
1 J/cm²	98.0 %± 1.4	99.0 %± 1.2	98.0 %± 1.4
2 J/cm²	99.0% ± 3.2	103.0% ± 1.2	99.0%± 1.3

Alizarin red staining was performed in order to see bone mineralization nodules at 7^{th} and 14^{th} day. The micrographs show that all groups showed mineral deposits (Figure 4.20). The orange dots inside the cells are calcium nodules that are stained by the dye. Figure 4.21 shows the normalized absorbance at 405 nm in order to quantify Alizarin red staining. At day 14, all the groups had significantly decreased calcium depositions compared to the control group ($p<0.05$) but no differences in mineralization levels have been observed at day 7.

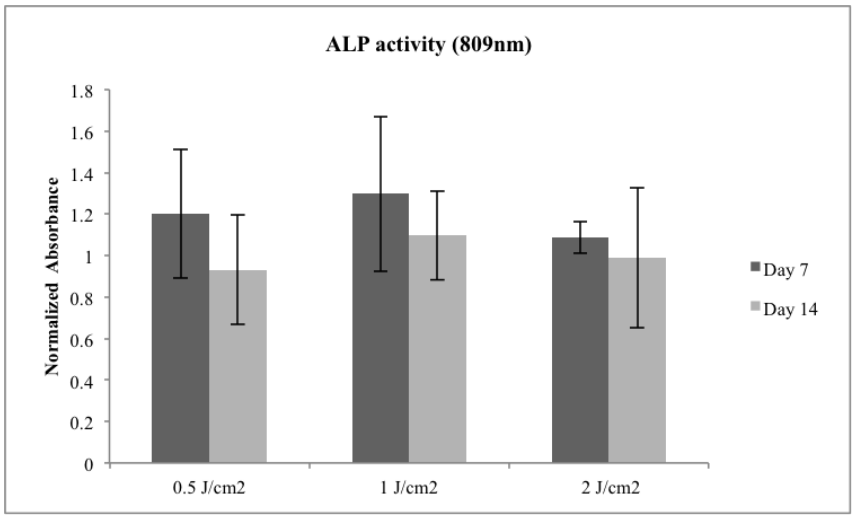

Figure 4.19 Normalized absorbance showing ALP activity. No statistical difference between groups was observed ($p>0.05$).

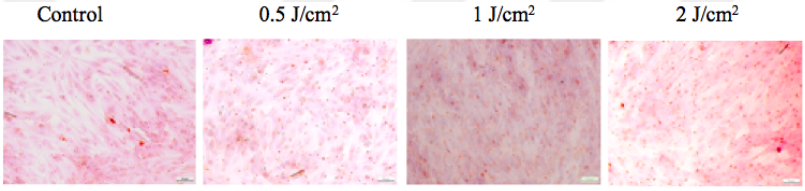

Figure 4.20 Microscopic images of osteoblasts (10x) treated with different doses of laser irradiation at day 14 with Alizarin red. Scale bar: 100 μm.

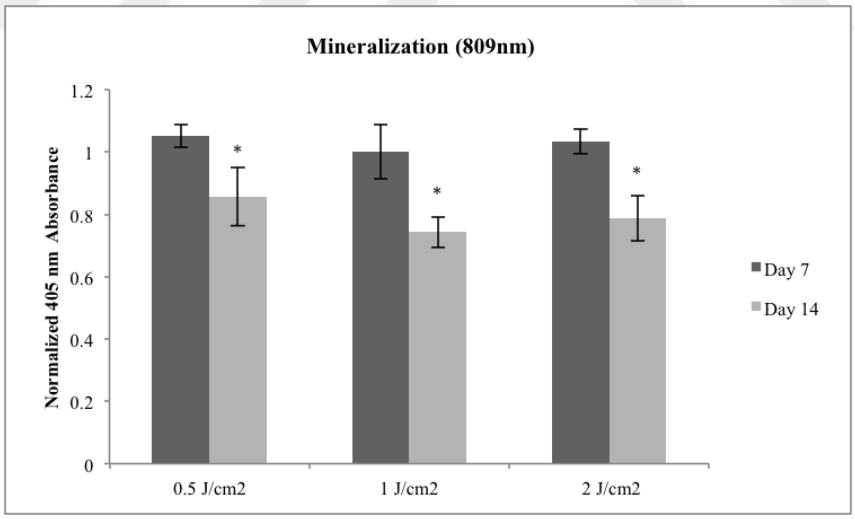

Figure 4.21 Normalized absorbance at 405 nm after Alizarin red staining. At day 14, all the groups had significantly decreased calcium depositions compared to the control group. * indicates statistically significant difference ($p<0.05$).

4.3.3 Discussion

The relative gene expressions normalized to GAPDH at 7^{th} and 14^{th} day after laser application are given in Figures 4.22. At day 7, all groups had significantly de-

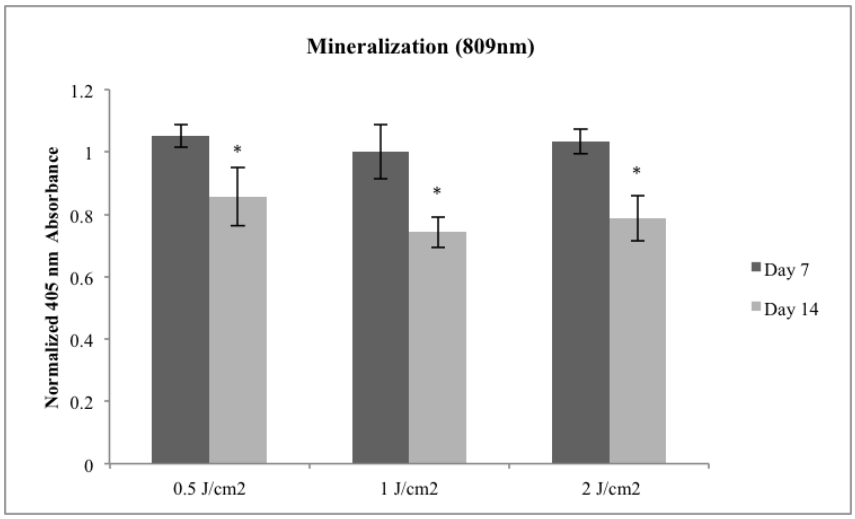

Figure 4.22 The relative gene expressions normalized to GAPDH at 7^{th} and 14^{th} day. * indicates statistically significant to the control group ($p<0.05$).

creased BGALP expression. On the other hand, ALPL expression was increased in all experimental groups. The COL1A synthesis was not affected by the treatment. RT-PCR data of 809 nm laser irradiation at day 14 revealed that 0.5 J/cm^2 irradiation did not altered none of these gene expressions. Cells irradiated with 1 and 2 J/cm^2 showed a significant decrease in BGLAP expression at 14^{th} day. ALPL expression was not affected by 809 nm laser irradiation. The only laser dose that significantly increased COL1A expression was 1 J/cm^2 group.

The results demonstrate that overall there were no differences on cell viability and proliferation between control and experimental groups after a single laser irradiation according to the MTT, Alamar Blue and AO/PI staining results. A transient effect was found at end of 48 h according to the MTT assay for 809 nm irradiation but the results of the Alamar Blue proliferation assay or AO/PI staining did not reveal data for supporting this effect. The energy doses used in this study were chosen similar to those in several previous studies that investigated PBM. The results of the present study are in agreement, with several reports from different groups that showed no significant biostimulatory effect on cell proliferation. The effects of laser irradiation at 830 nm (0.5, 1, 5 and 10 J/cm^2) and 780 nm (0.5, 1, 5 and 10 J/cm^2) on proliferation and ALP activity of normal primary osteoblasts (MC3T3) were investigated by Renno et al. Low level irradiation at 830 nm produced a statistically significant increase in osteoblast proliferation only at a dose of 10

J/cm^2. However, 780 nm irradiation caused statistically significant decrease in osteoblast proliferation at doses of 1, 5 and 10 J/cm^2 [58]. The same group did another research using a diode (830nm) with a single exposure at 10 J/cm^2 on osteoblastic (MC3T3) cell line using bioscaffolds, but this time a reduction in cell growth and proliferation was observed when cells were grown on glassceramic discs [59].

Conflicting results also exist in the measurement of ALP activity and mineralization in response to PBM. Coombe et al. reported that no significant early or late effects were observed on protein expression or ALP activity when the osteosarcoma cells (SAOS-2) were irradiated as a single or daily dose up to 10 days with a 830 nm laser [60]. Petri et al. stated that low-level laser treatment at 3 J/cm^2 (780 nm) did not influence culture growth, ALP activity, and mineralized matrix formation of on human osteoblastic cells grown on Titanium [61]. Similarly, a study by Khadra et al.,the human osteoblast-like cells exposed to 1,5 and 3 J/cm^2 (830 nm) did not show statistically significant change in ALP activity [62]However, Medina-Huertas et al. who evaluated the effect of 940 nm laser irradiation on osteoblast-like cells (MG-63) and concluded that doses of 1W/cm^2/3 J, 1W/cm^2/4 J, or 1.5 W/cm^2/3 J produced a major and significant increase in ALP activity [63]. In another study, the human fetal osteoblast cells were measured for the ALP activity at days 1, 3, and 7 after 940 nm irradiation (for a period of up to 7 days using different power values) and an increase in ALP activity was observed in all groups [64]. In the study of Ozawa et al. ALP activity was significantly stimulated on days 9–18 after laser irradiation on days 1, 6, 12, and 15 (830 nm/3.82 J/cm^2) [65].

Several *in vivo* studies suggest that PBM with wavelengths in the infrared region may have positive effect on bone growth. In their study, Weber et al. used PBM (830 nm, 10 J/cm^2) trans-operatively and showed a positive effect on the healing of bone defects associated with autologous bone grafts [66]. Another group suggested that the positive effect of PBM was more evident when light was applied on the surgical bed intraoperatively, prior to the placement of the autologous bone graft [67]. Hübler et al. evaluated and found a positive effect of PBM (830 nm, 10 J/cm^2) chemical composition and crystalline structure of bone at the site of distraction osteogenesis [68].

It can be stated that PBM with 809 nm light at 0.5, 1.0 and 2.0 J/cm^2 energy densities caused a transient increase in cell viability at end of 48 h, increased ALPL gene expression at day 7 but inhibited mineralization at 14^{th} day. Osteocalcin (BGLAP protein) is involved in binding calcium during the mineralization process. Its decreased expression may have affected mineralization. Infrared light may have shown biostimulative effects in literature but that conclusion cannot be drawn definitely from the results of the present study.

4.4 STUDY 4: Indocyanine green mediated photobiomodulation (809nm) on human osteoblasts

4.4.1 Aim

The purpose of the present study is to investigate the effect of ICG-mediated PBM on osteoblast cells. The effect of 809 nm laser light at three different energy densities (0.5, 1.0 and 2.0 J/cm^2) combined by ICG application on the osteoblast proliferation and behavior is examined by measuring cell viability, proliferation, ALP activity, mineralization, ROS production, and gene expressions of phenotypic osteoblast markers (ALPL, BGLAP, COL1A).

4.4.2 Results

The dark toxicity of ICG was assessed by MTT assay at 24, 48 and 72h. Among concentrations between 0.5–20.0μM, none of them caused significant cell death within the first 72h (Figure 4.23) The smallest concentration (0.5μM) was chosen to be used for light combined experiments.

According to the MTT results, cell viability at 24h was significantly increased for ICG+2 J/cm^2 group compared to the control group ($p<0.05$). It appears to have a slight increasing trend in ICG+1 J/cm^2 group but the difference was not significant. At

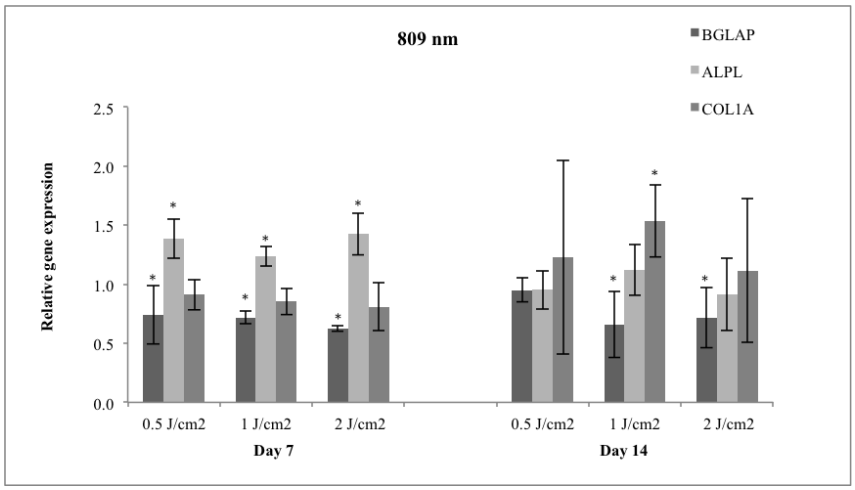

Figure 4.23 Cell viability percentages of osteoblasts treated with different concentration of ICG. None of the concentration showed significant cell death ($p>0.05$) compared to the control.

72h, both ICG+1 J/cm^2 and ICG+2 J/cm^2 groups had significantly decreased viability compared to the control group ($p<0.05$). However, the level of the cell death did not reach lethal dose 50%, it was about 15% (Figure 4.24).

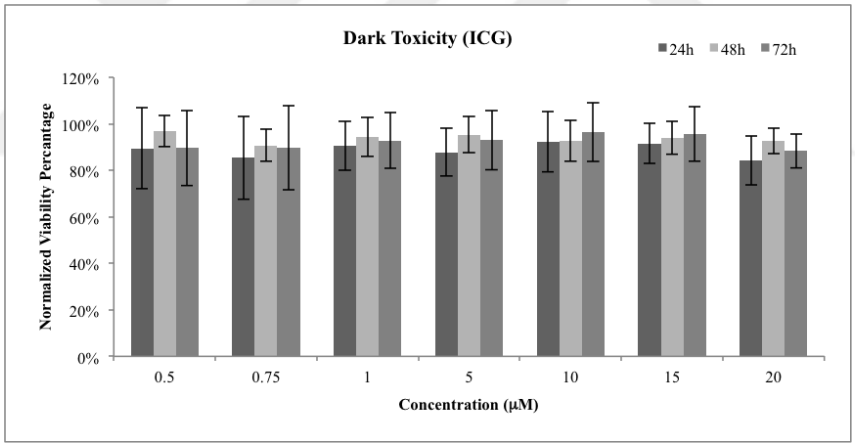

Figure 4.24 Normalized absorbance at 570 nm for MTT assay performed 24,48 and 72 h after treatment. ICG concentration was 0.5 μM. * indicates statistically significant difference ($p<0.05$) compared to the control group.

The Alamar Blue assay results showed that the experimental groups did not differ from each other at any assay time (Fig. 4.25).

AO/PI staining was performed in order to visualize under fluorescent microscope and quantify live/dead cells by ImageJ software. The data are given in Table 4.4. No statistical difference was found when experimental groups are compared to control group ($p>0.05$).

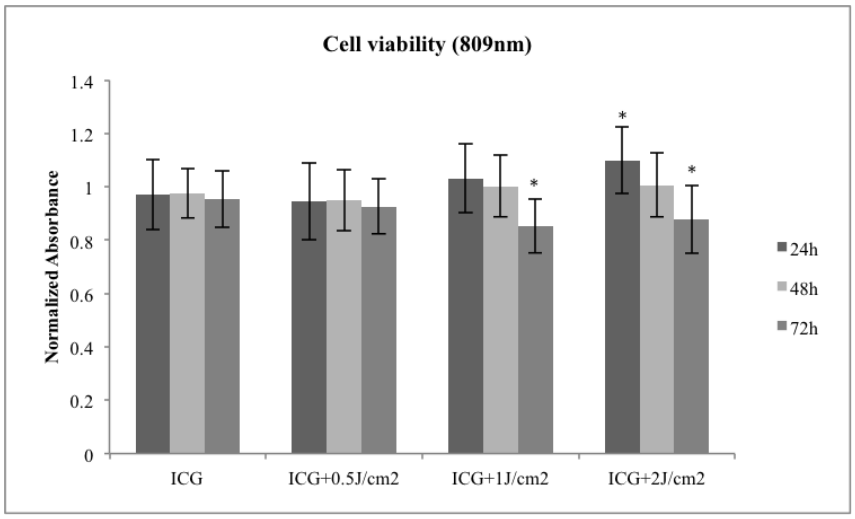

Figure 4.25 Normalized absorbance at 570 nm for MTT assay performed 24,48 and 72 h after treatment. ICG concentration was 0.5 μM. * indicates statistically significant difference ($p<0.05$) compared to the control group.

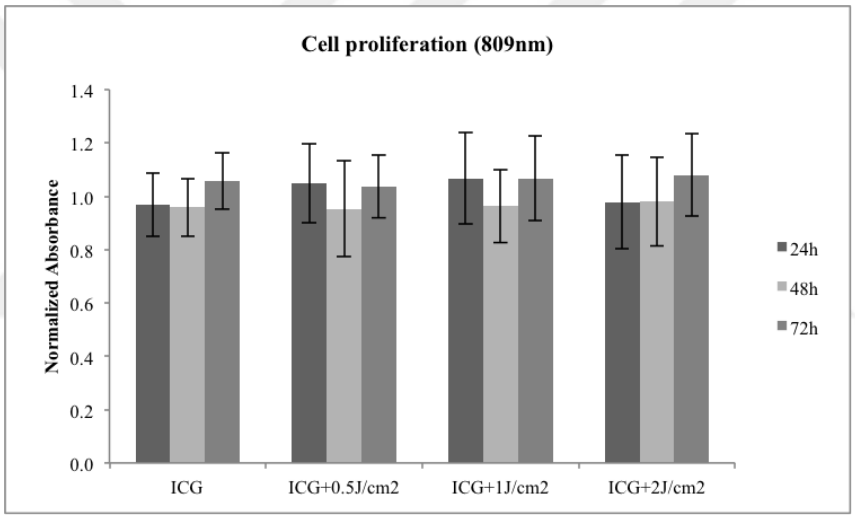

Figure 4.26 The normalized optical densites of Alamar Blue assay at 24, 48 and 72 h. ICG concentration was 0.5 μM. No significant differences were observed between groups ($p>0.05$).

The ALP activity was measured at 7^{th} and 14^{th} day after irradiation. All experimental groups at both time periods did not have any significant change in ALP activity. Although an increasing trend was observed in laser-combined groups at day 7, the change was not statistically significant.

Mineralization was assessed at 7^{th} and 14^{th} day after ICG application and laser irradiation (Figure 4.27). When compared to the control groups, only ICG and ICG combined laser groups showed similar trends: the amount of mineralization was lower at

Table 4.4
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ. No differences were observed between laser groups and control cells.

809 nm	**24 h**	**48 h**	**72 h**
ICG	98.0 %± 2.2	99.0%± 1.8	99.0% ± 1.1
ICG+0.5 J/cm^2	98.0% ± 1.5	97.0%± 1.2	100.0% ± 1.6
ICG+1 J/cm^2	96.0% ± 2.7	99.0% ± 1.5	99.0% ± 3.2
ICG+2 J/cm^2	101.0% ± 2.9	98.0 %± 1.9	99.0% ± 2.6

7^{th} day, and higher at 14^{th} day ($p<0.05$).

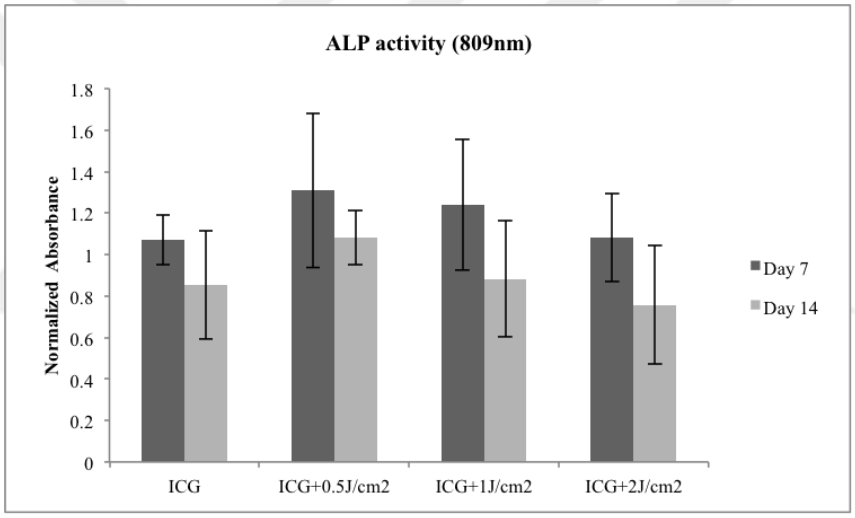

Figure 4.27 Normalized ALP activity measured at 7^{th} and 14^{th} day. All ICG-mediated PBM groups have tendency to have increased ALP activity at day 7. At 14^{th} day, all groups have similar ALP activity when compared to the control group.

The expressions of three genes: BGALP, ALPL and COL1A were measured by RT-PCR analysis at 7^{th} and 14^{th} days. The relative gene expressions are given in Figure 4.28. ICG+0.5 J/cm^2 group had significantly increased expression for BGALP but ICG+1 J/cm^2 group had decreased expression for the same gene at 7^{th} day ($p<0.05$). For 14^{th} day, all groups had similar expressions. ALPL expressions were higher than the control group at both days for all groups except ICG group at 7^{th} day and ICG+2 J/cm^2 group at 14^{th} day. ICG+2 J/cm^2 group had the highest expression at 7^{th} day for ALPL. All light combined ICG groups had significantly higher expressions for COL1A at 14^{th} day

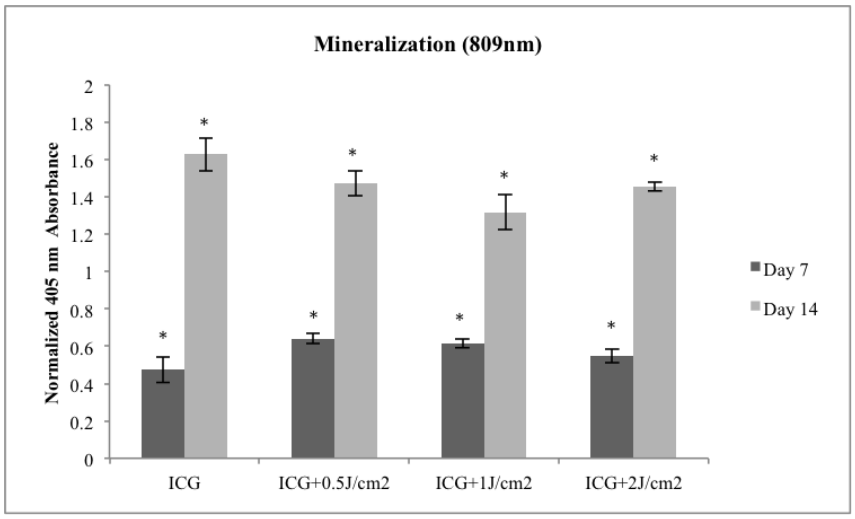

Figure 4.28 Normalized absorbance at 405 nm after Alizarin Red staining. ICG concentration was 0.5 μM. All of the treatment groups had significantly lower mineralization compared to the control group at 7^{th} day but higher mineralization at 14^{th} day ($p<0.05$).

($p<0.05$). At 7^{th} day, only ICG+1 J/cm^2 group showed increased expression of COL1A ($p<0.05$).

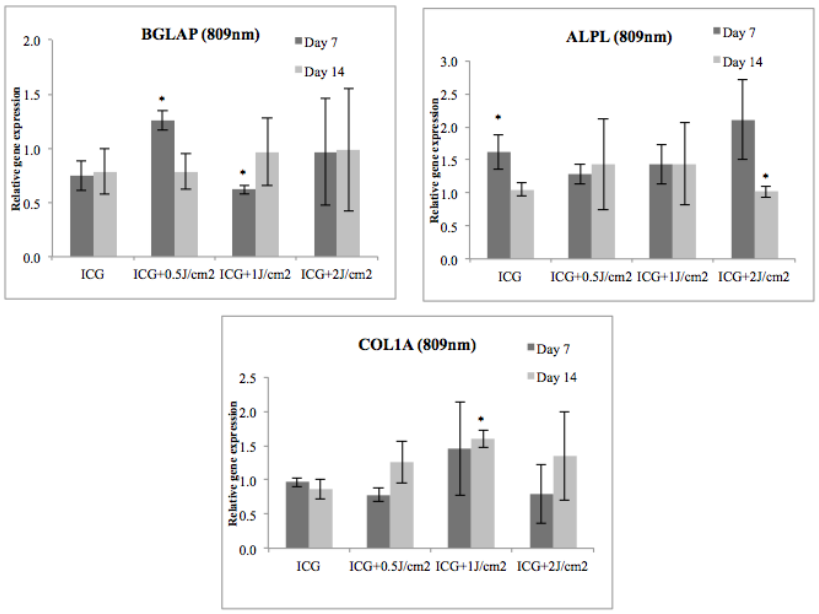

Figure 4.29 The relative gene expressions normalized to GAPDH at 7^{th} and 14^{th} day. * indicates statistically significant to the control group ($p<0.05$).

With the aim of investigating the mechanisms by which PS-mediated PBM could affect osteoblast behavior, singlet oxygen generation was performed using the trap molecule 1,3-diphenylisobenzofuran (DPBF) in ethanol. ICG application did not alter the absorbance peak of DPBF so it did not created singlet oxygen. When the light at 809 nm is

combined with ICG, singlet oxygen production started and absorbance of DPBF, which peaks at 414 nm decreases. As energy density increases, the decrease in DPBF absorbance increases (Figure 4.29) meaning singlet oxygen generation increases. The presence of ROS has been demonstrated also by ROS fluorescence staining (Figure 4.30).

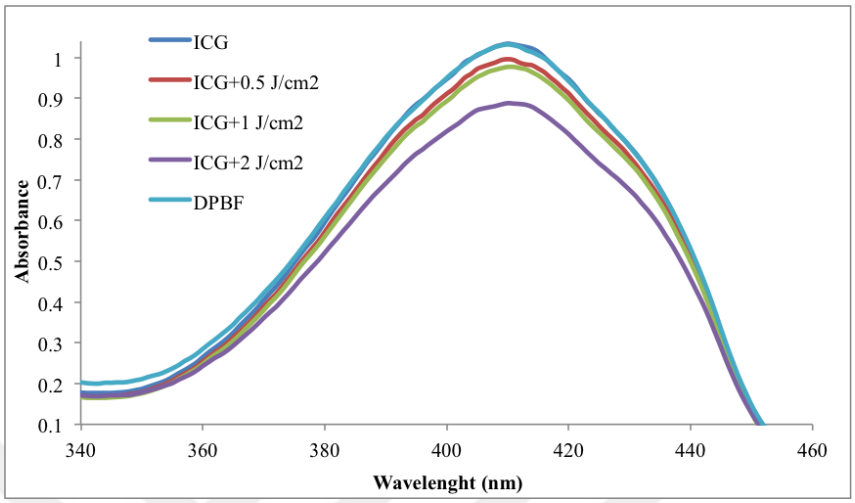

Figure 4.30 Decrease in absorbance spectrum of DPBF after ICG and laser irradiation.

4.4.3 Discussion

The photosensitizing properties of ICG make it a suitable agent for PDT studies. Several reports have indicated that ICG do not exhibit dark toxicity for concentrations up to even 500 μM [28,30]. Since we wanted to deliver less amount of drug to the cells, we have assessed dark toxicity of ICG concentrations between 0.5 and 20 μM and similar to the literature, we have found that ICG did not cause cell death at 24, 48 and 72h after its application according to MTT assay results. The chosen ICG concentration was 0.5 μM for ICG-mediated PBM experiments. Suggested mechanisms to explain the cell proliferation by PBM states that when red and near-infrared light is absorbed by mitochondrial respiratory chain components the resulting increase in the amount of ROS, adenosine triphosphate (ATP)/or cyclic AMP would initiate a signaling cascade and finally cellular proliferation and cytoprotection will be promoted [69]. In order to show ROS production by laser combined IGC application, fluorescent ROS staining was performed. When cells were subjected to 809 nm laser irradiation after ICG application, the amount fluorescence increased. Moreover, testing singlet oxygen generation by using DPBF have shown that

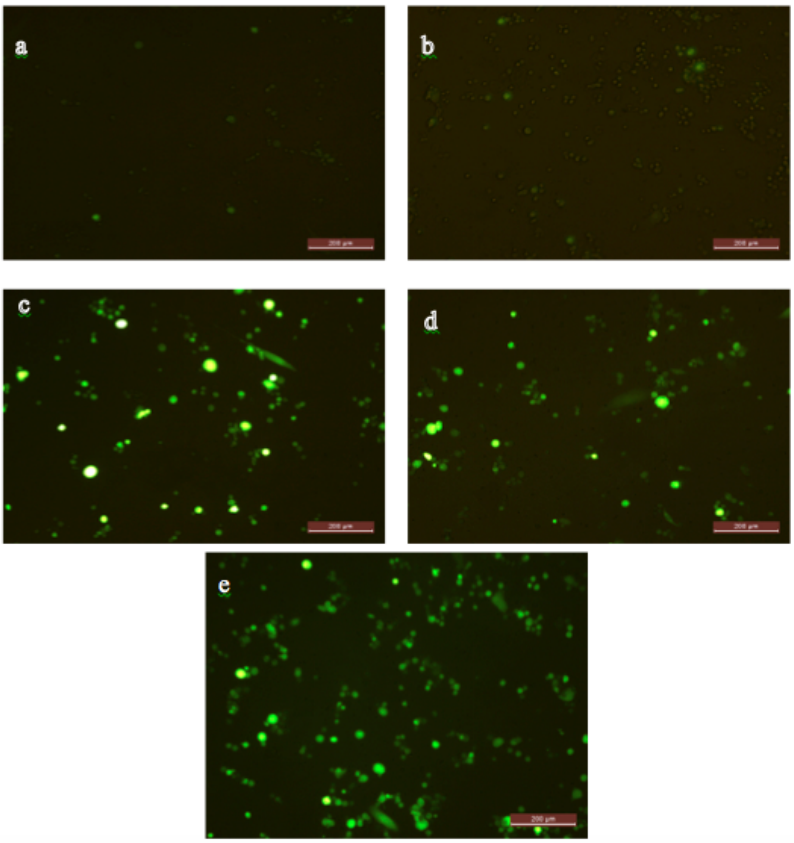

Figure 4.31 Micrographs showing ROS staining after ICG and laser irradiation. Scale bar:200 μm. a) control, b) ICG, c)ICG+0.5 J/cm^2, d) ICG+1 J/cm^2, e)ICG+2 J/cm^2

singlet oxygen is generated when 0.5 μM ICG is irradiated by 809 nm light at 0.5, 1 and 2 J/cm^2 energy densities.

The cell viability and proliferation was measured by three different assays: MTT, Alamar Blue and AO/PI staining. There were no increase in cell numbers between control and experimental groups 24, 48 or 72h after irradiation except a slight increase in ICG+2 J/cm^2 group at 24h, which is only detected by MTT assay. Again MTT assay results revealed that at 72h, both ICG+1 J/cm^2 and ICG+2 J/cm^2 groups had significantly decreased viability compared to the control group ($p<0.05$). However cell viability was greater than 90% in all experimental and control groups, no drastic inhibitory effect was observed. In the previous study, it was shown that cell viability increased at 48h post-irradiation when 0.5,1 and 2 J/cm^2 809 nm laser is applied [54]. However, with ICG application prior to laser, this effect seems to be disappeared or occurred early around 24h.

Not only the cell viability but also metabolic activities may be altered by PBM applications. The anabolic activity of osteoblasts can be examined by measuring their ALP activity, mineralization capacity and related gene expressions. The results of the present study showed that ICG-mediated PBM enhanced mineralization of existing osteoblasts at 14^{th} day. The mineralization was significantly decreased at 14^{th} day when 809 nm laser was applied to osteoblast as shown on the previous study [54]. However, when PBM is combined with ICG, the effect is reversed. It significantly increased mineralization at day 14.

There are few studies that investigated the combination of PS and PBM to increase cell activity. Stein et al. used human osteosarcoma cell line SaOS-2 subjected to 670-nm irradiation at energy densities of 1 J/cm^2 or 2 J/cm^2 to investigate the effects of PBM followed by pre-treatment with phenothiazine chloride. They stated that PS combined PBM did not demonstrate any additional synergistic effect compared to PBM alone [70]. Zancanela et al. used aluminum phthalocyanine chloride (AlClPc) as PS and combined it to 670 nm irradiation at 0.5, 1 and 3 J/cm^2 to stimulate osteoblasts, which were isolated from the bone marrow of rats. They observed a slight increase in cell viability only for 0.5 J/cm^2 group [71].

These results may contribute to low-PDT studies in which cell damage is aimed. Careful investigations should be done since ICG combined laser irradiation may have some stimulating effects.

4.5 STUDY 5: The effects of photobiomodulation (635nm) on osteoblasts differentiated from adipose-derived stem cells

4.5.1 Aim

The purpose of this study was to investigate the effects of 635 nm laser light on the osteogenic differentiation of ASCs. Cell viability, proliferation, ALP activity, mineral-

ization and gene expressions of phenotypic osteoblast markers (ALPL, BGLAP, COL1A) were assessed.

4.5.2 Results

To evaluate the effect of PBM on cell viability, MTT assay was performed on day 7 and 14. The analysis of normalized optical absorbance values has shown that 635 nm laser irradiation has no effect on cell viability on day 7 and 14, except for 0.5 J/cm^2 group at 14^{th} day ($p<0.05$).

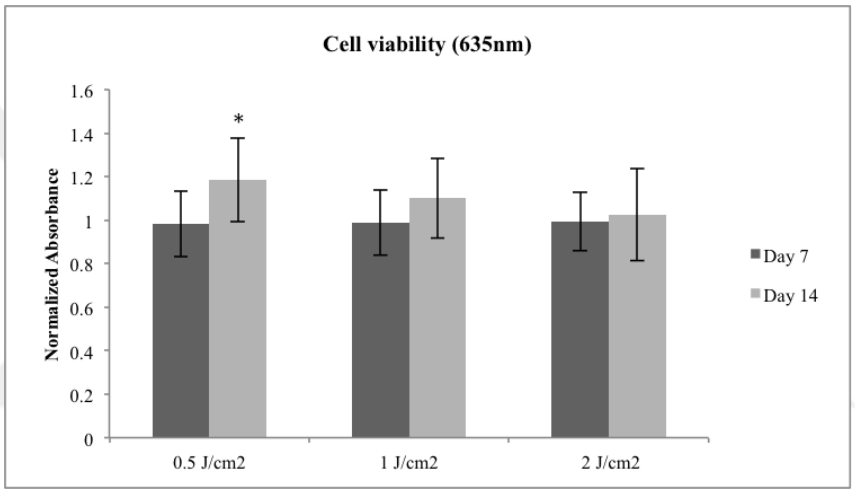

Figure 4.32 Normalized absorbance at 570 nm for MTT assay. 0.5 J/cm^2 group at 14^{th} day had significantly increased cell viability. * indicates statistically significant difference ($p<0.05$).

Alamar Blue assay of ASCs cultured in ODM, which reflects the proliferation rate, showed that irradiation at 635nm did not cause any significant change in proliferation (Figure 4.32).

AO/PI staining was performed in order to visualize under fluorescent microscope and quantify live/dead cells by ImageJ software. The data are given in Table 4.5.No statistical difference was found when experimental groups are compared to control group ($p>0.05$).

The ALP activity of the cells irradiated with 635 nm did not show any differences from the control group at day 7 and 14 (Figure 4.33).

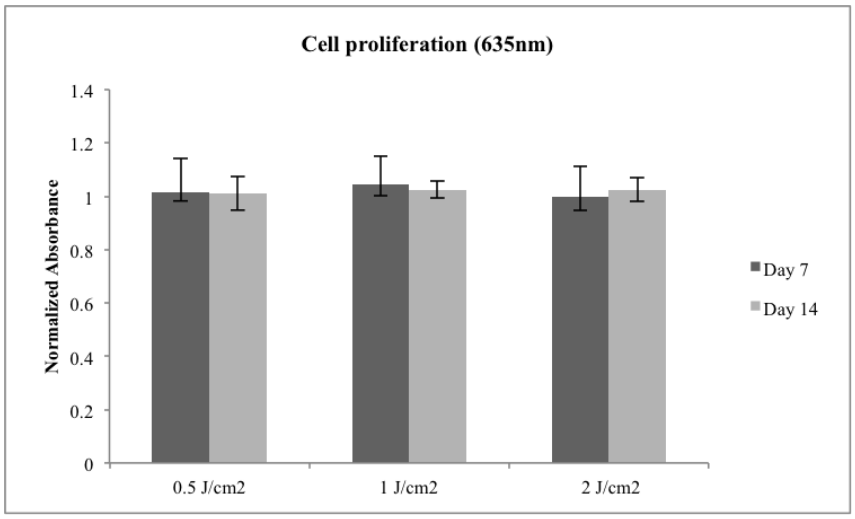

Figure 4.33 Normalised absorbance for Alamar Blue assay at days 7 and 14 after laser irradiation. No significant differences were recorded ($p>0.05$).

Table 4.5
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ. No differences were observed between laser groups and control cells.

635 nm	**Day 7**	**Day 14**
0.5 J/cm²	101.0% ± 1.1	99.0% ± 2.7
1 J/cm²	97.0% ± 2.3	103.0% ± 0.8
2 J/cm²	98.0% ± 0.8	96.0% ± 1.7

Microscopy analysis of cells cultured in ODM stained with Alizarin Red at 14^{th} day has demonstrated many nodules of calcium in the mineralized extracellular matrix whereas cells cultured in DMEM were not stained with Alizarin Red showing that they were not differentiated into osteoblasts (Figure 4.34b and 4.34c). 635nm irradiation at 0.5 J/cm^2 results were not different than the control group. The mineralization level was statistically decreased for 1 J/cm^2 group at day 14. The 2 J/cm^2 group had statistically decreased mineralization at day 7 but increased mineralization at day 14 (Figure 4.34a)

The effect of PBM on the expression of three osteoblast markers, namely BGLAP, ALPL and COL1A were analyzed by RT-PCR. The chart in Figure 4.35 compares the relative gene expressions at 7 and 14 days after irradiation with 635 nm laser. At 7^{th}

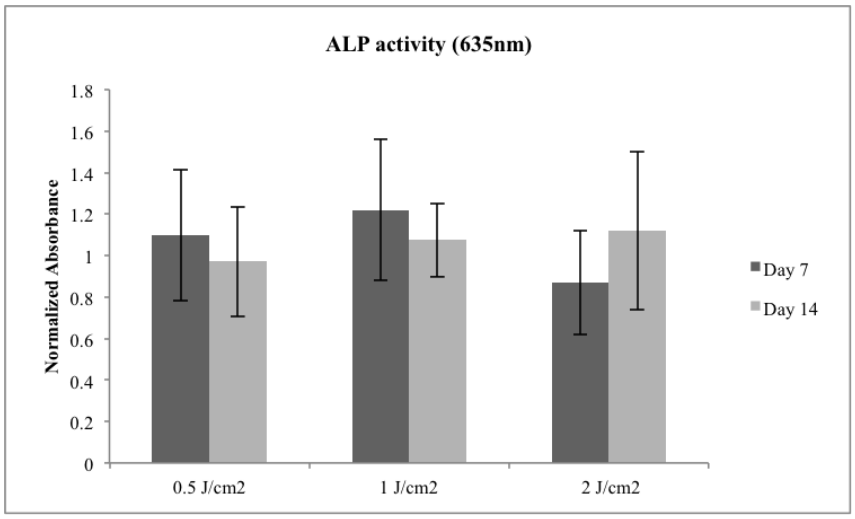

Figure 4.34 Normalized ALP activity at 7^{th} and 14^{th} day after irradiation. Although there were slight increases for 1 J/cm^2 group ay day 7, the differences were not statistically significant ($p>0.05$).

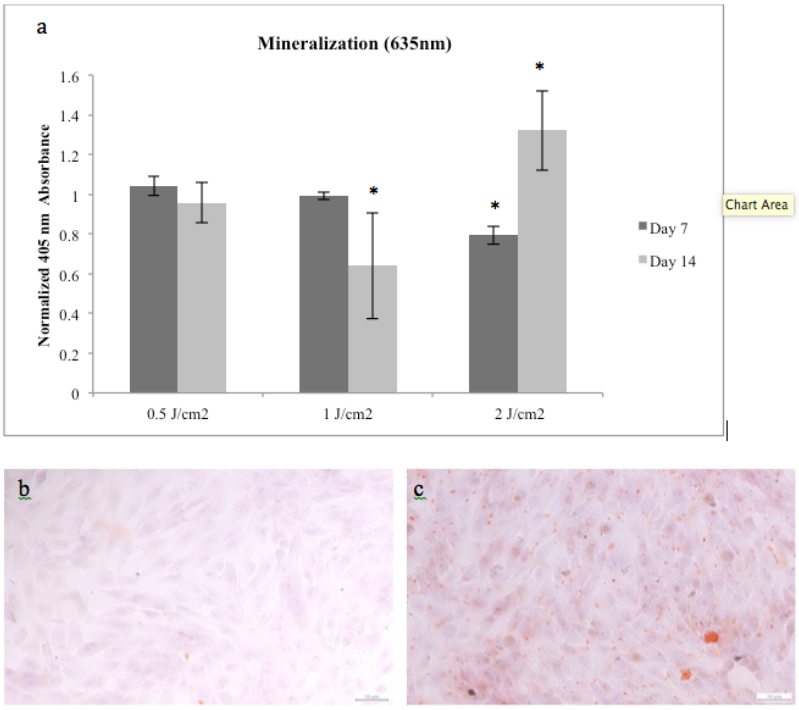

Figure 4.35 (a) normalized absorbances for mineralization. * indicates statistically significant difference compared to the control ($p<0.05$). Microscopic images (10x) of two different controls. (b) cells cultured in DMEM , (c) cells cultured in ODM. Small orange dots are calcium deposits. Cells cultured in DMEM were not stained with Alizarin Red showing that they were not differentiated into osteoblasts.

day, similar expression patterns of BGLAP and ALPL were observed for the laser and control groups. COL1A expression was statistically lower in the 1J/cm^2 group at 7^{th} day. ALPL expression is slightly increased at 14^{th} day in all groups although no statistically significance was recorded. BGLAP expressions were slightly lower for all groups, but

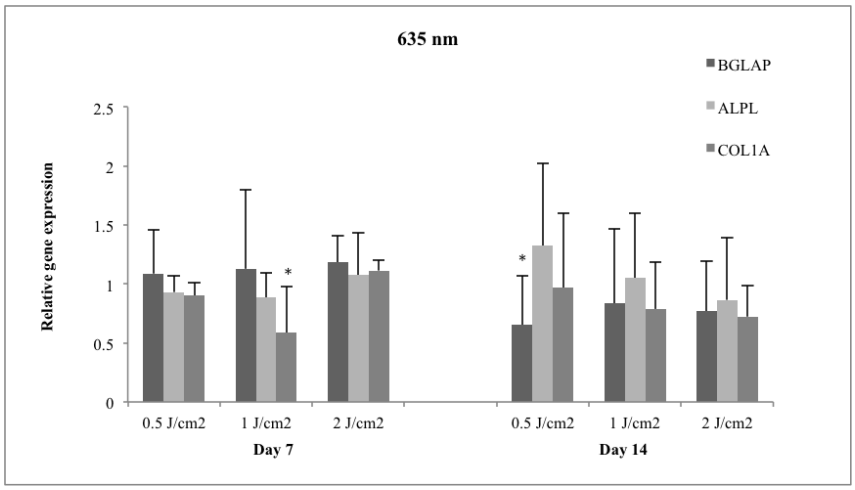

Figure 4.36 The relative gene expressions normalized to GAPDH at 7^{th} and 14^{th} days after 635 nm laser irradiation on ASCs * indicates statistically significant compared to the control group ($p<0.05$).

statistically significant for only 0.5 J/cm^2 group. COL1A expressions were similar when compared to the control group at 14^{th} day.

4.5.3 Discussion

To summarize, the only significant difference in cell viability was observed for 0.5 J/cm^2 group at 14^{th} day and Alamar Blue assay results of the present study showed cell proliferation was significantly affected by 635 laser irradiation at 0.5, 1 and 2 J/cm^2 after 7 and 14^{th} day. Similarly, ALP activity assessed at same experimental days pointed out that laser irradiation at stated parameters did not affect ALP activity. ALPL mRNA expressions were slightly but not significantly higher at 14^{th} day for all laser groups. BGLAP and Col1a expressions were not affected at overall by laser irradiation. Mineralization data did not reveal any meaningful outcome unfortunately.

There are studies investigating proliferation of ASCs in DMEM [21, 69–73] but there are not many studies using ASCs differentiating into osteoblasts to show the effect of PBM. Moreover, usually mesenchymal stem cells (MSCs) were used in PBM studies. Therefore, it is difficult to compare the results. Nevertheless, studies carried out with other sort of stem cells such as MSCs were considered to discuss the results. A study performed by Peng et al. revealed that red light delivered every other day (LED, 620 nm,

1,2 and 4 J/cm^2) suppressed proliferation of MSCs cultured in ODM but enhanced their osteogenic differentiation. The RT-PCR results showed that the Col1a1 and ALP mRNA expressions in red light irradiated cultures did not significantly differ from the control groups, but BGLAP expression levels were significantly increased on day 4 [74]. Kim et al. exposed mouse MSCs to a LED light of 647 nm for 10 s, 30 s or 90 s with radiation energies of 0.093 J, 0.279 J and 0.836 J, respectively. Their results showed that cell viability was not affected by irradiation but ALP activity, mineralization, osteocalcin and collagen1 mRNA expression was increased compared to the control groups [75]. In 2012, Saygun et al. used 685 nm laser irradiation (once or twice at 2 J/cm^2) on osteoblasts differentiated from MSCs and stated that irradiation have increased proliferation and release of growth factors [76].

Investigation of the effects of the 3D biomatrix/ 632.8 nm laser light combination (0.5 mW/cm^2) on the modulation of MSCs into an osteogenic lineage showed that ALP activity was significantly enhanced in early stages and notably reduced in late stages of culturing [77].

Osteoblast differentiation may be divided into three stages. In the first two stage, within 1 or 2 weeks, cells slowly proliferate, express ALP activity and bone specific genes, and produce a collagen matrix. Mineralization is an endpoint step which reflects advanced cell differentiation occurring in week 2 or 3 [57]. Studies suggest that mineralization should be accompanied by increased expression or activity of osteocalcin and alkaline phosphatase [16]. Light irradiation may result in shortening of all differentiation process. At 14^{th} day, differentiation process has been completed and mineralization is high compared to the control group. Therefore, ALP activity may have also peaked earlier than expected that we do not observe any peak at 14^{th} day. It should also be noted that ALP is synthesized as an inactive precursor and activated by proteolytic cleavage [33–35,78]. When ALP activity is measured, it is its active form whereas mRNA expressions reflect inactive amounts. Light may also intervene in activation process, altering the proteolytic cleavage.

As a result, irradiation at 635 nm with 0.5 J/cm^2 energy density increased cell number at 14^{th} day after irradiation. The effect may be more prominent if the cells were

subjected to multiple irradiations.

4.6 STUDY 6: The effects of MB-mediated photobiomodulation (635nm) on osteoblasts differentiated from adipose-derived stem cells

4.6.1 Aim

The aim of this study was to investigate the effects of MB-mediated PBM on differentiation of ADS into the osteogenic lineage. The ADS were treated with MB for 1 h and irradiated with 809 nm laser light at energy densities of 0.5, 1.0 and 2.0 J/cm^2. After irradiation, the cells were incubated in osteogenic medium in order to differentiate them into osteoblasts. Cell viability, proliferation, ROS generation, ALP activity, mineralization and gene expression of osteoblast markers were assessed in order to investigate the effects of MB-mediated PBM on differentiation of ADSs.

4.6.2 Results

First step was to determine the non-toxic concentration of MB to be used with light combined experiments. According to the MTT results performed at 24,48 and 72h after 1 h incubation with MB, among the concentration between 0.05 and 10 μM, the 0.05 μM was chosen to combine with laser experiments in order to deliver less photosensitizer to the cells (Figure 4.36). Concentrations higher than 1 μM caused significant cell death ($p<0.05$).

MTT assay was performed after 7 and 14 day of irradiation (Figure 4.37). The number of cells was similar in all groups and the control group at all time points studied ($p>0.05$).

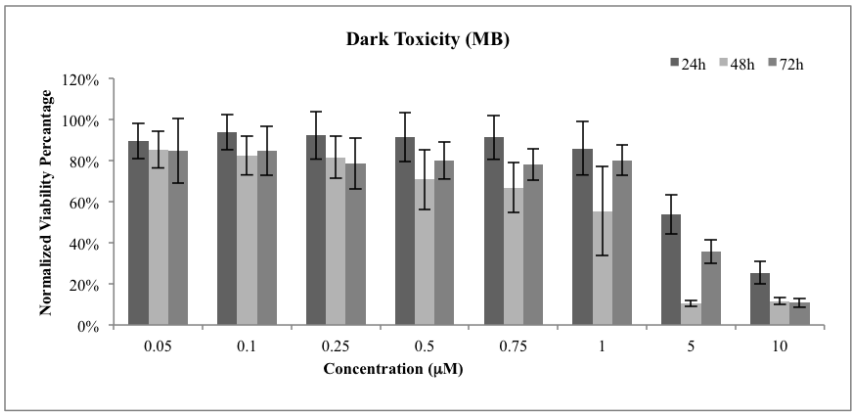

Figure 4.37 Cell viability of ASC treated with different concentration of methylene blue. The concentration higher than 1μM showed more than 50% cell death ($p<0.05$) compared to the control group.

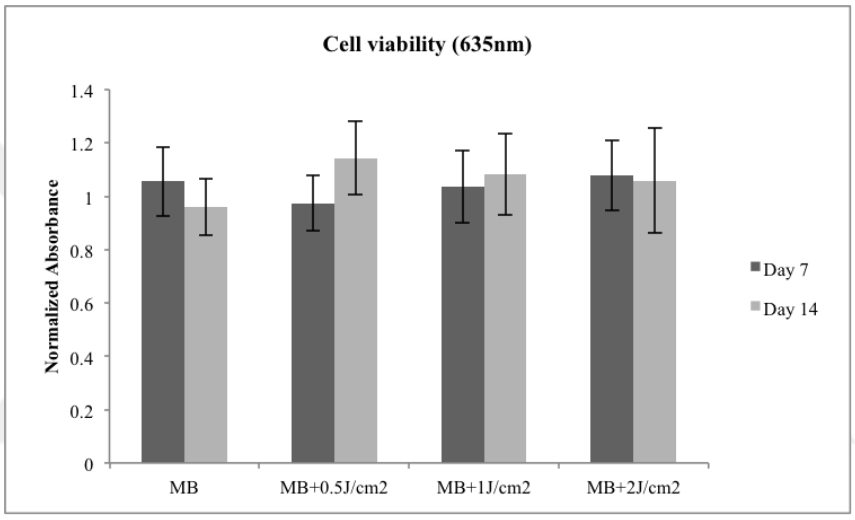

Figure 4.38 Normalized absorbance for MTT assay at days 7 and 14 after laser irradiation. There were no significant differences between groups ($p>0.05$).

Proliferation activity was determined by means of the Alamar Blue Assay at day 7 and 14. No statistical differences were revealed between experimental and control groups ($p>0.05$).

AO/PI staining was performed in order to visualize under fluorescent microscope and quantify live/dead cells by ImageJ software. The data are given in Table 4.6. No statistical difference was found when experimental groups are compared to control group ($p>0.05$).

Although there is an increasing trend in ALP activity of all groups at day 7, no statistically significant difference was observed (Figure 4.39). On the other hand, a

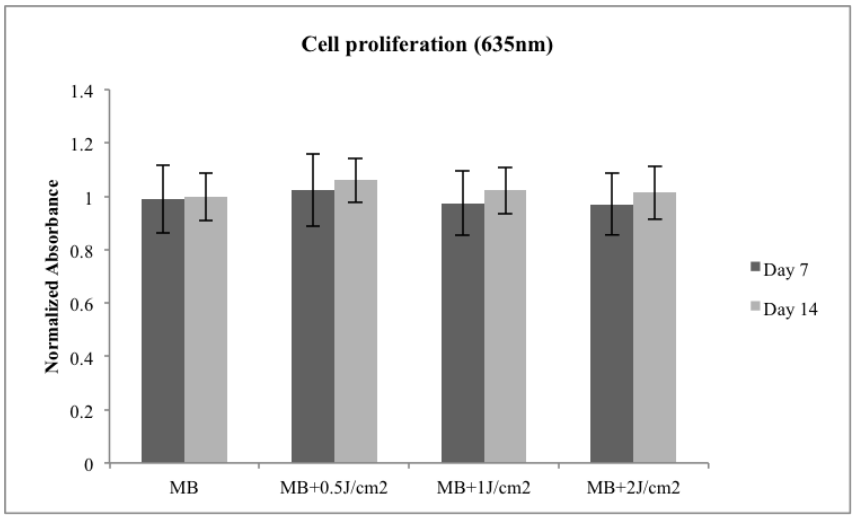

Figure 4.39 The normalized absorbance for Alamar Blue assay, which measures proliferation at 7^{th} and 14^{th} day. No significant differences were observed between groups ($p>0.05$).

Table 4.6
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ. No differences were observed between laser groups and control cells.

635 nm	**Day 7**	**Day 14**
MB	99.0% ± 2.6	88.0% ± 5.8
MB+0.5 J/cm^2	104.0% ± 1.3	95.0% ± 2.3
MB+1 J/cm^2	97.0%± 1.4	102.0% ± 1.4
MB+2 J/cm^2	89.0% ± 2.2	96.0% ± 2.5

significant increase in ALP activity of all groups at day 14 was recorded ($p<0.05$).

Alizarin Red staining was done to detect calcium depositions. All cells were positive for Alizarin Red staining with orange calcium nodules indicating osteogenic differentiation when examined under microscope. Quantification of the staining have shown that all laser combined MB groups seemed to have decreased mineralization, but significant only for MB+0.5 and MB+2 J/cm^2 groups at day 7 and MB+1 J/cm^2 group at day 14. Interestingly only MB group increased mineralization at day 7 (Figure 4.40).

The effect of PBM on the expression of three genes: BGLAP, ALPL and COL1A were analyzed by RT-PCR (Figure 4.41) at 7^{th} and 14^{th} day after MB-treated irradiation.

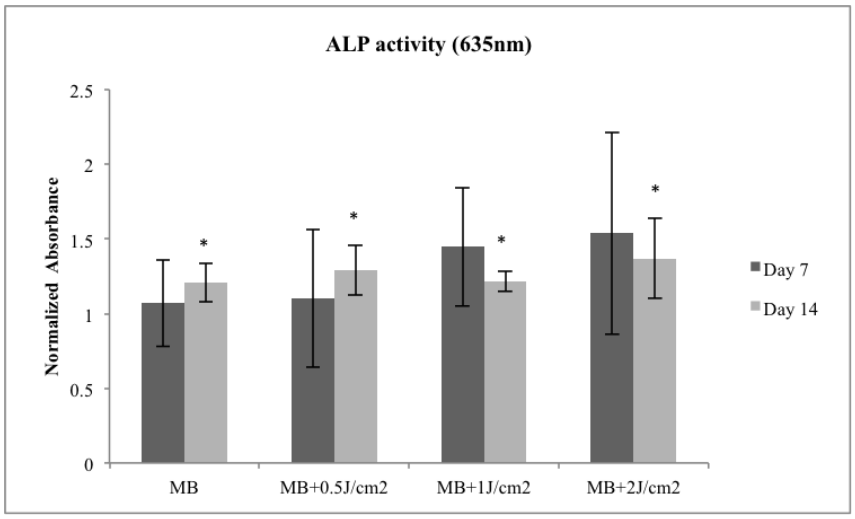

Figure 4.40 Normalized ALP activity at 7^{th} and 14^{th} day after irradiation. * indicates statistically significant difference compared to the control ($p<0.05$).

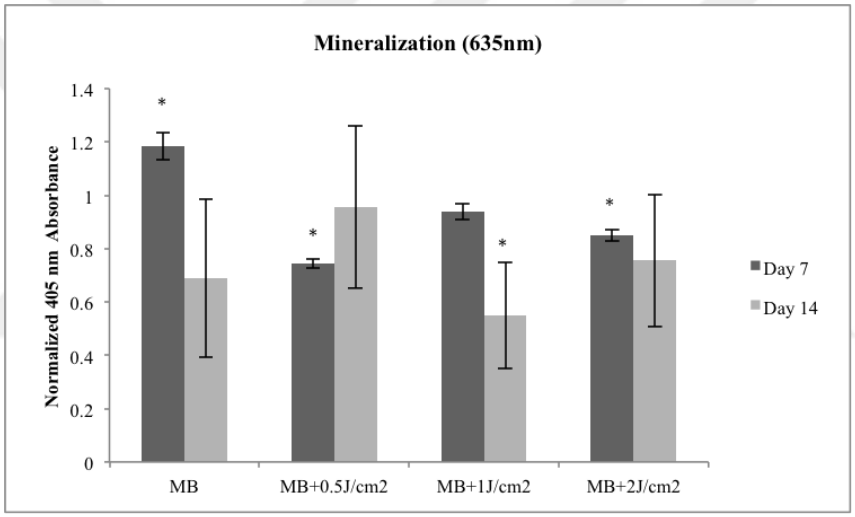

Figure 4.41 Normalized absorbance at 405 nm for mineralization. * indicates statistically significant difference compared to the control ($p<0.05$).

MB and MB+ 0.5 J/cm^2 groups had significantly increased ALPL expression. For other gene expressions at day 7 or at day 14, no statistical differences were observed.

4.6.3 Discussion

MB-mediated 635 nm irradiation did not affected cell viability or proliferation within 14 days. However, ALP activity was significantly increased for all groups at day 14. This is correlated with increased ALPL expression in MB+0.5 J/cm^2. However,

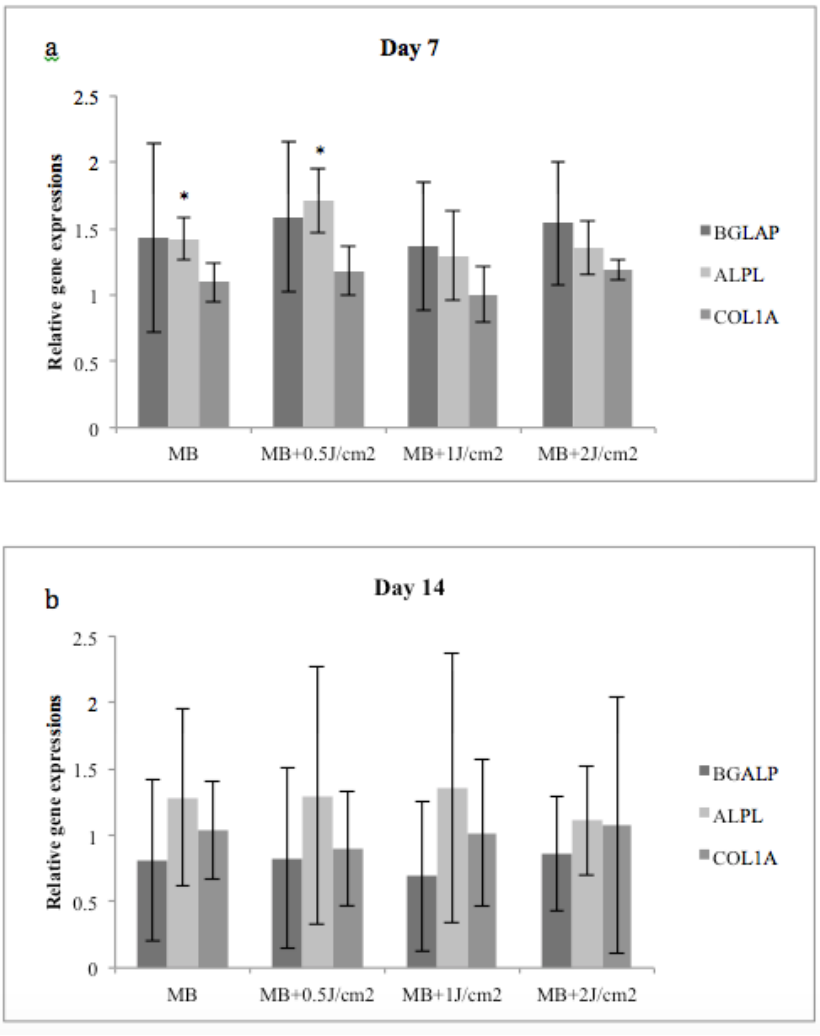

Figure 4.42 RT-PCR analysis at 7^{th} (a) and 14^{th} (b) day for indicated genes. * indicates statistically significant difference compared to the control ($p<0.05$).

the increase in ALP did not increased mineralization at 14^{th} day. Since mineralization is the end point, later measurement (at 21^{th} day or 28^{th}) may have shown increased mineralization in the groups with higher ALP activity at day 14.

Moreover, studies suggest that level of ALP activity may not be proportional to mineralization levels. The molarities of calcium and phosphate in the culture media can influence the collagen matrix mineralization. For example, 3.0μM inorganic phosphate (Pi) or 5.0–10.0 mM disodium β-glycerol phosphate (βGP) is necessary to initiate *in vitro* mineralization. In the presence of 2mM calcium and 2mM phosphate, however, the collagen matrix is formed but it is not mineralized [79] Inhibition of ALP or low level of it may result in cultures with non-mineralized collagen matrix [47, 48]. Levels of

dexamethasone is another factor that may influence mineralization [57]. The increased level of ALP but decreased level of mineralization may have resulted because of chemical changes in cell culture medium.

A few studies have been performed asking the same question whether combined PS and laser application would cause cell proliferation or inhibition. Zancanela et al. investigated the effect of 670 nm laser (at 0.5, 1 and 3 J/cm^2) mediated by nanoemulsions of photosensitizer drug aluminum phthalocyanine chloride (AlClPc) on osteoblast growth [80]. They have observed that when delivered AlClPc is combined with 0.5 J/cm^2, the cell viability, total protein, ALP activity and collagen increased significantly indicating enhanced cell growth. On the other hand, irradiation at 3 J/cm^2 in the presence of AlClPc led to the reduction in the same parameters, therefore showing phototoxicity. In another *in vitro* study, human immortalized keratinocyte growth was efficiently stimulated by photodynamic treatment with low methyl 5-aminolevulinate concentrations [3] .

Several *in vivo* studies also showed positive results when light is combined with PS. A group of authors used δ-aminolevulinic acid (ALA) along with He-Ne laser [81] . Their results suggested that animals that received ALA and He-Ne laser showed the quickest wound-healing pattern. Hamblin et al. claimed that the photodynamic treatment did not damage the host tissue when it was able to kill Escherichia coli infecting excisional wounds in mice [82]. Another study that use PS and low level laser was conducted by Silva et al. on rats using an InGaAIP diode laser at 685 nm mediated by Chloro-Aluminium phthalocyanine-derived PS. They indicated that association of light and a PS does not inhibit but can improve healing process [83]. The data of another study performed by Xhang et al. indicate that low-dose PDT results in increased VEGF expression and endothelial cell proliferation in normal brains of nude mice [2]. These studies with the positive effects of PDT, which actually aim to damage the target tissue, show another time that the parameters should be determined carefully. It should be noted that the results could be completely reversed upon light type or energy density. On the other hand, these studies also show that photodynamic effect can be used to produce conditions to modulate cellular function and induce cell proliferation.

4.7 STUDY 7: The effects of photobiomodulation (809nm) on osteoblasts differentiated from adipose-derived stem cells

4.7.1 Aim

The purpose of this study was to evaluate the behavior and metabolism of cells osteoblasts differentiated from adipose-derived stem cells subjected to 809 nm laser irradiation.

4.7.2 Results

Cell viability analyzed by the MTT assay method was similar in the control and irradiated groups at all time points studied (Figure 4.42).

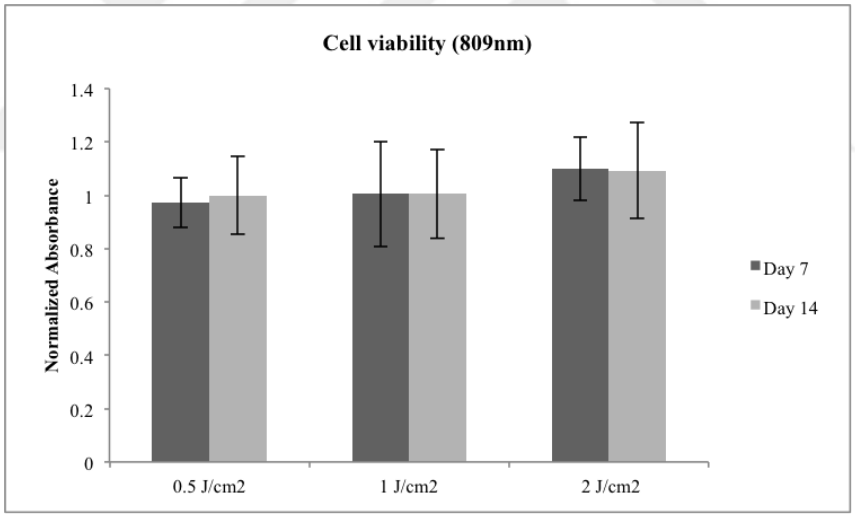

Figure 4.43 Normalized absorbance for MTT assay at days 7 and 14 after laser irradiation. There were no significant differences between groups ($p>0.05$).

Alamar Blue assay of ASCs cultured in ODM, which reflects the proliferation rate, showed that irradiation at 809 nm did not cause any significant change in proliferation ($p>0.05$) (Figure 4.43).

AO/PI staining was performed in order to visualize under fluorescent microscope and quantify live/dead cells by ImageJ software. The data are given in Table 4.7.No

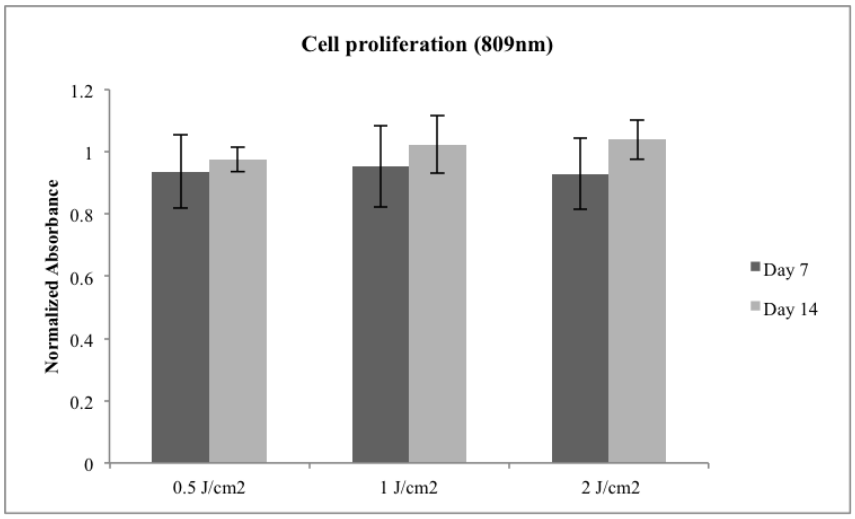

Figure 4.44 The normalized absorbance for Alamar Blue assay, which measures proliferation at 7^{th} and 14^{th} day. No significant differences were observed between groups ($p>0.05$).

Table 4.7
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ. No differences were observed between laser groups and control cells.

809 nm	**Day 7**	**Day 14**
0,5 J/cm²	105.0% ± 2.5	97.0% ± 1.9
1 J/cm²	99.0% ± 0.9	99.0% ± 1.5
2 J/cm²	97.0% ± 2.1	103.0% ± 2.3

statistical difference was found when experimental groups are compared to control group ($p>0.05$).

Although there was a slight increase in ALP activity of cells irradiated with 809 nm (1 J/cm^2 and 2 J/cm^2), no statistical differences were observed between control and laser groups at both time intervals (Figure 4.44).

Cells subjected to 809 nm irradiation at all energy densities showed statistically increased mineralization, 1 J/cm^2 group being the least ($p<0.05$). Microscopy analysis of cells cultured in ODM stained with Alizarin Red at 14^{th} day has demonstrated many nodules of calcium in the mineralized extracellular matrix whereas cells cultured in DMEM were not stained with Alizarin Red showing that they were not differentiated

into osteoblasts.

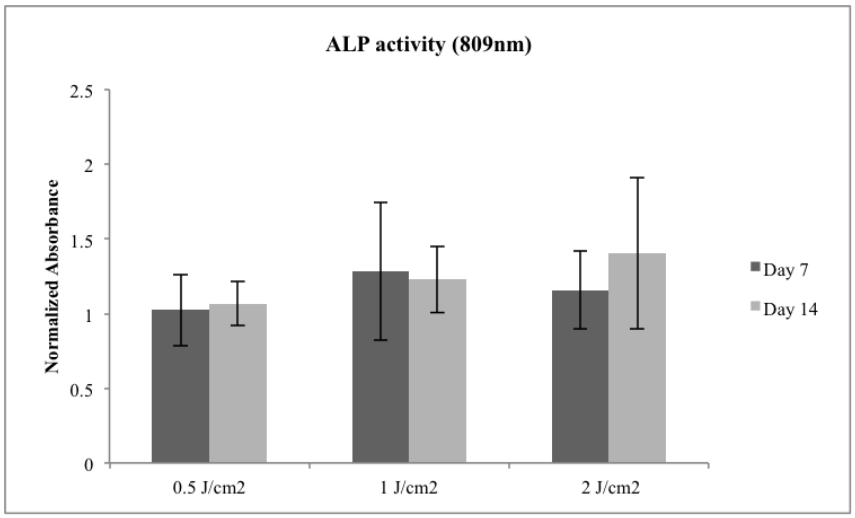

Figure 4.45 Normalized ALP activity at 7^{th} and 14^{th} day after irradiation. * indicates statistically significant difference compared to the control ($p<0.05$).

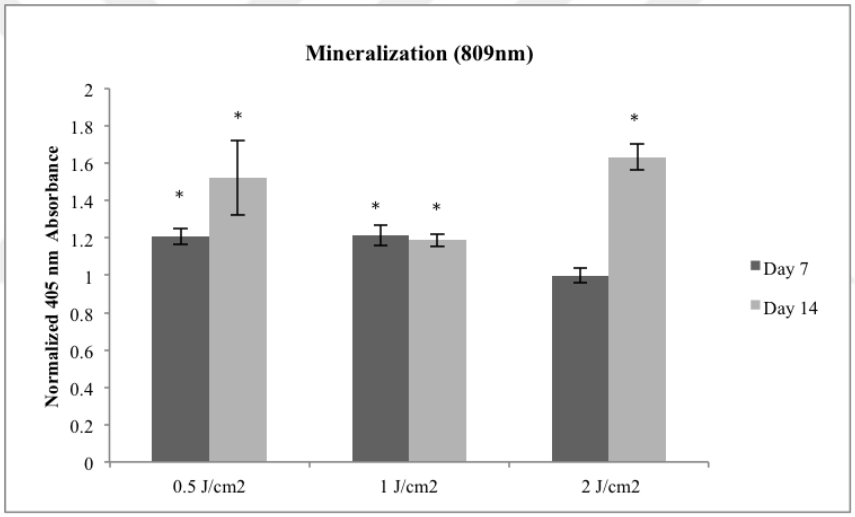

Figure 4.46 Normalized absorbance at 405 nm for mineralization. * indicates statistically significant difference compared to the control ($p<0.05$).

RT-PCR analysis data are given in Figure 4.46. In 0.5 and 1 J/cm^2 groups, ALPL and COL1A expressions were down regulated at day 7. However, ALPL expressions were slightly but not significantly higher at 14^{th} day for all laser groups. BGLAP and COL1A expressions were not affected at overall by laser irradiation at 14^{th} day.

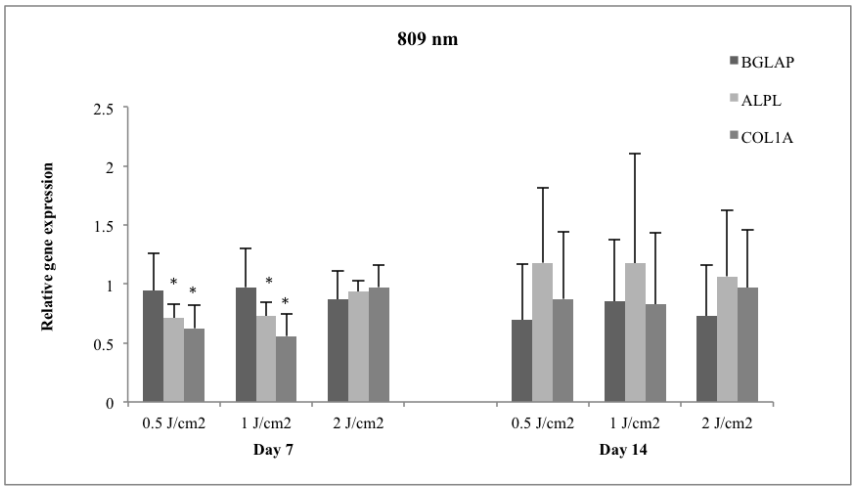

Figure 4.47 RT-PCR analysis of bone specific genes at 7^{th} and 14^{th} day. * indicates statistically significant difference ($p<0.05$) compared to the control group.

4.7.3 Discussion

The MTT assay, Alamar Blue assay and AO/PI staining results of this study showed that neither cell viability nor cell proliferation was significantly affected by 809 nm laser irradiation at 0.5, 1 and 2 J/cm^2 after 7 and 14^{th} day. Similarly, ALP activity assessed at same experimental days pointed out that laser irradiation at stated parameters did not affect ALP activity. However, mineralization at 14^{th} day was increased after 809 nm laser application except for 2 J/cm^2 group at 7^{th} day. In 0.5 and 1 J/cm^2 groups, ALPL and COL1A expressions were down regulated at day 7. Hoever, ALPL expressions were slightly but not significantly higher at 14^{th} day for all laser groups. BGLAP and COL1A expressions were not affected at overall by laser irradiation at 14^{th} day.

Studies suggest that mineralization should be accompanied by increased expression or activity of osteocalcin and alkaline phosphatase [84]. Mineralization at 14^{th} day was higher for all 809 nm laser groups. Our RT-PCR results for the same groups showed that ALPL expressions were slightly higher at 14^{th} day although not significant. However, osteocalcin (BGLAP) expressions did not differ from the control group. It seems that light irradiation did not altered differentiation process but it may have caused shortening of all differentiation process.

There are not many studies using ASCs differentiating into osteoblasts to show the effect of PBM. The ones using red light were discussed in the previous parts. Soleimani et al. used bone marrow-derived mesenchymal stem cells (BMSCs) to investigate the effect of 810 nm laser application at 2 or 4 J/cm^2 on their differentiation to osteoblasts [23]. They irradiated cells at days 1, 3 and 5 after incubation of BMSCs with ODM and concluded that PBM increases BMSC proliferation and its differentiation to osteoblasts at the 7^{th} day. Moreover, ALP activities were significantly higher at both energy densities compared to the control group. On the other hand, 808 nm laser light at an energy density of 4 J/cm^2 does not alter murine BMSCs proliferation and differentiation into osteoblasts according to the results of viability, specific staining assays and RT-PCR analysis for list of osteoblast markers [85].

The results of this study suggest that PBM with 809 nm wavelength has no inhibitory effect on cells, on the contrary, these parameters may be used if the aim is to increase mineralization of osteoblasts which are differentiated from ASCs.

4.8 STUDY 8: The effects of ICG-mediated photobiomodulation (809nm) on osteoblasts differentiated from adipose-derived stem cells

4.8.1 Aim

The purpose of this study was to figure out the effects of ICG-mediated PBM on ASCs commitment to osteoblast lineage. The ADS were treated with ICG for 1 h and irradiated with 809 nm laser light at energy densities of 0.5, 1.0 and 2.0 J/cm^2. After irradiation, the cells were incubated in ostegenic medium in order to differentiate them into osteoblasts. Cell viability, proliferation, ROS generation, ALP activity, mineralization and gene expression of osteoblast markers were assessed in order to investigate the effects of ICG-mediated PBM on differentiation of ADSs.

4.8.2 Results

Dark toxicity of different concentrations of ICG was tested by MTT assay at 24, 48 and 72h after incubation with ICG. None of the tested concentrations cause cell death, therefore the smallest concentration, which was 0.5 μM, was chosen to be used in laser combined experiments (Figure 4.47). This is the same concentration used in the osteoblast experiments.

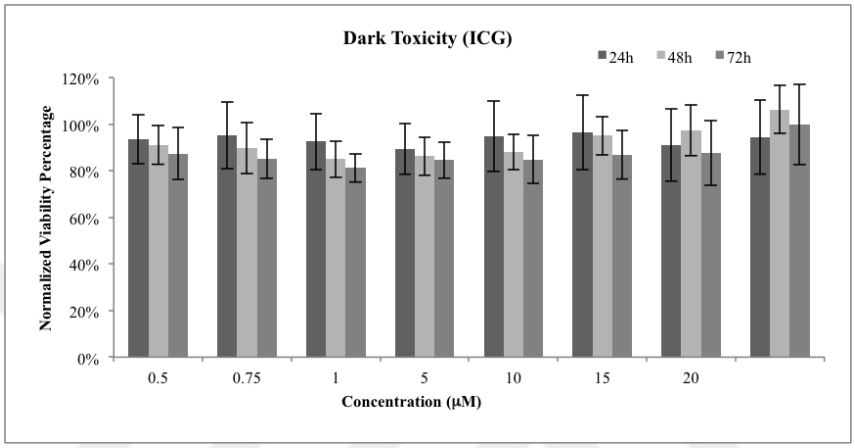

Figure 4.48 Cell viability percentages of osteoblasts treated with different concentration of ICG for 1h. None of the concentration showed significant cell death ($p>0.05$) compared to the control.

MTT assay was performed on ADSs cultured in ODM for up to 7 and 14 days to determine cell viability. No significant changes were observed when compared to the control group ($p>0.05$). Figure 4.48 shows the results concerning cell proliferation found in test samples. The proliferation level was similar to control group in all experimental groups showing no statistically significant changes.

The ALP activity of ICG+0.5 J/cm^2 and ICG+1 J/cm^2 groups were higher when compared to the control group at day 7. All groups had significantly increased ALP activity at 14^{th} day after irradiation (Figure 4.50)

The mineralization level of all groups at day 7 was similar with the control group. However, their mineralization was considerably higher when compared to untreated group at day 14 (Figure 4.51).

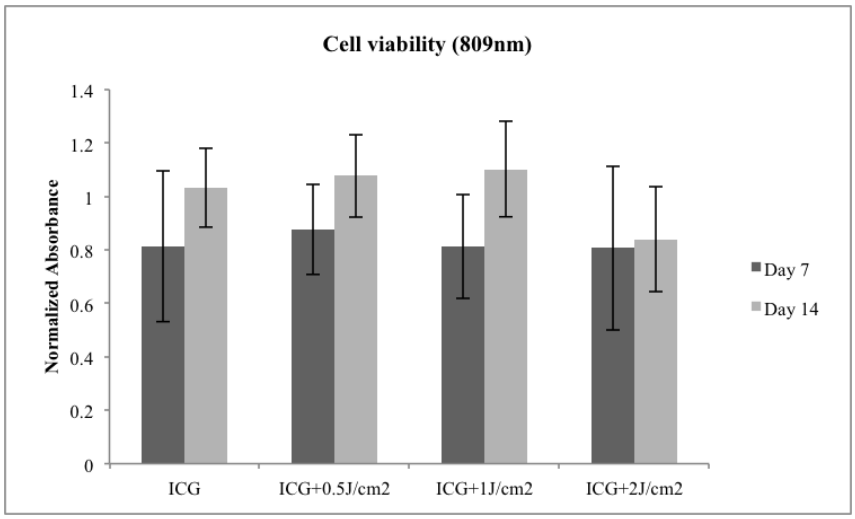

Figure 4.49 Normalized absorbance for MTT assay at days 7 and 14 after laser irradiation. ICG concentration was 0.5 μM. There were no significant differences between groups ($p>0.05$).

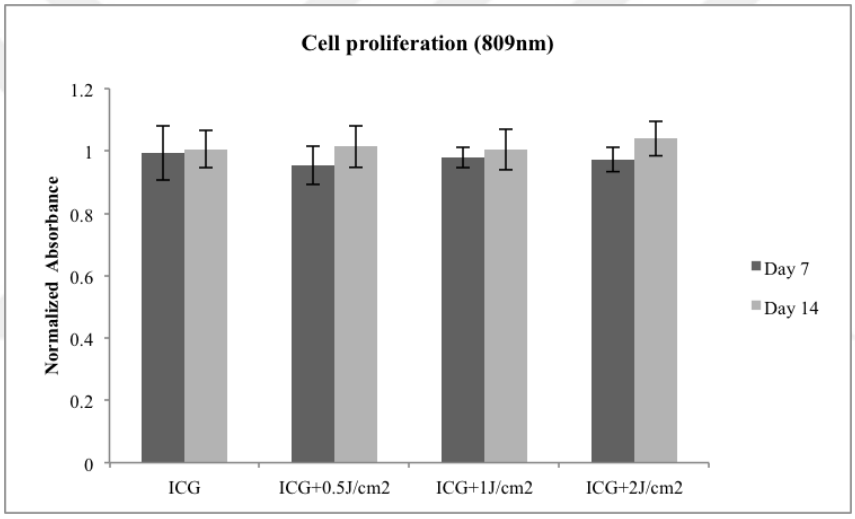

Figure 4.50 Cell viability percentages of osteoblasts treated with different concentration of ICG for 1h. None of the concentration showed significant cell death ($p>0.05$) compared to the control.

In order to characterize bone specific gene expressions, RT-PCR analysis was performed at 7^{th} and 14^{th} days (Figure 4.52). There was a statistically significant down regulation of BGLAP at both time points for ICG group. No evident changes were detected in other experimental groups

Table 4.8
Percentages of viable cells according to AO/PI staining results. The micrographs were quantified by ImageJ. No differences were observed between laser groups and control cells.

809 nm	**Day 7**	**Day 14**
ICG	98.0% ± 1.6	96.0% ± 3.5
ICG+0.5 J/cm^2	99.0% ± 2.5	99.0% ± 1.9
ICG+1 J/cm^2	102.0% ± 3.7	98.0% ± 0.8
ICG+2 J/cm^2	101.0% ± 2.2	100.0% ± 2.3

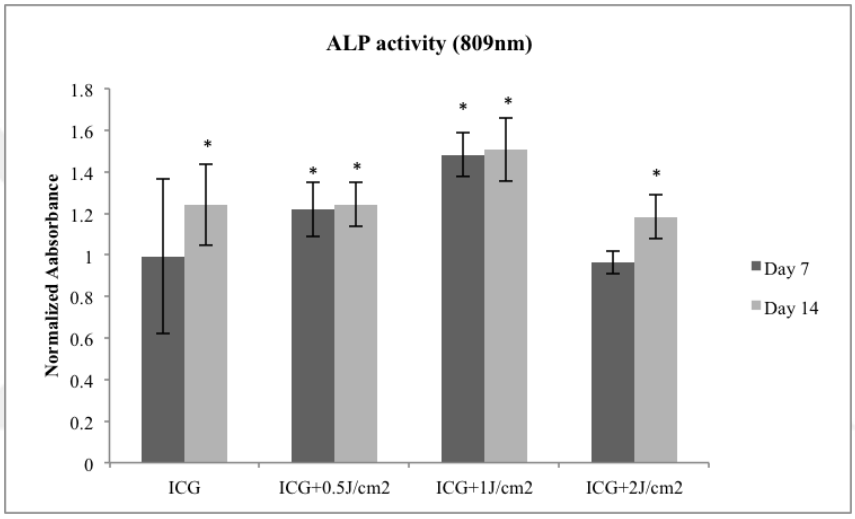

Figure 4.51 Normalized ALP activity measured at 7^{th} and 14^{th} day. ICG concentration was 0.5 μM. * indicates statistically significant difference ($p<0.05$) compared to the control group.

4.8.3 Discussion

Even though, ICG mediated PBM did not cause any evident change in cell viability or proliferation, ALP activity and mineralization was significantly affected. Nevertheless, the same biostimulative effect was not confirmed by RT-PCR analysis results.

Throughout the differentiation process, in the first 1-2 weeks cells slowly proliferate, express ALP activity and form the extracellular collagen matrix. The 2^{nd} and 3^{th} week is the maturation phase during which mineralization of the matrix is marked [57]. This may explain why cell number did not change by ICG-mediated PBM but ALP activity

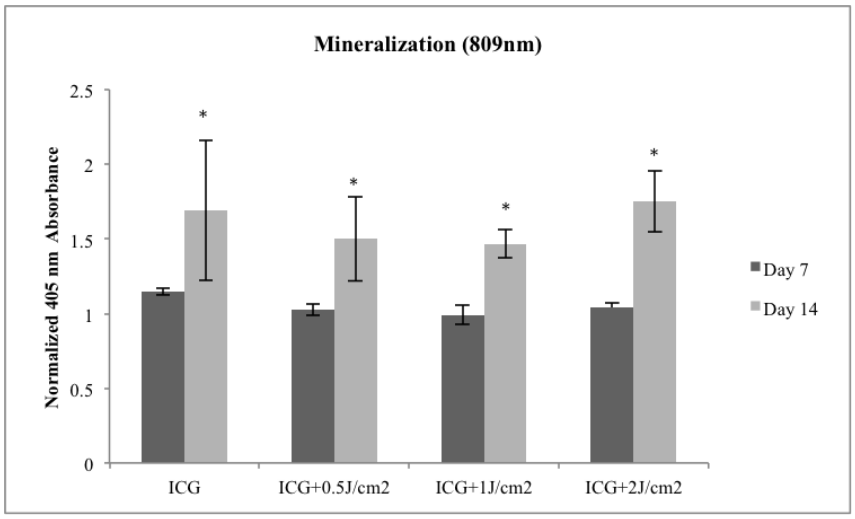

Figure 4.52 Normalized absorbance at 405 nm after Alizarin Red staining. ICG concentration was 0.5 μM. All ICG-laser groups have significantly increased mineralization at day 14. * indicates statistically significant difference ($p<0.05$) compared to the control group.

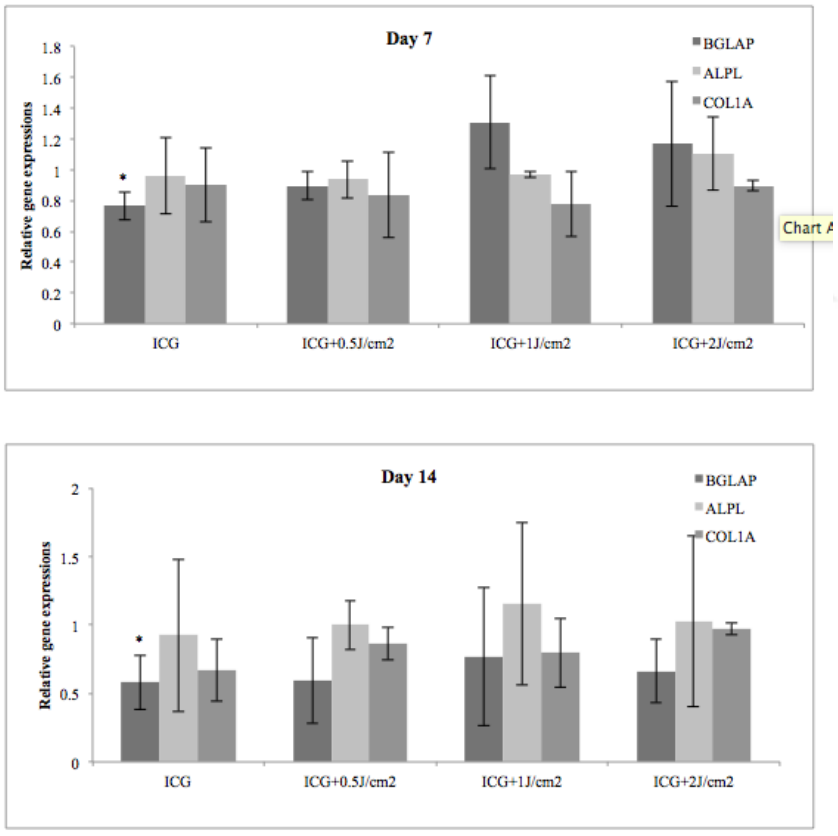

Figure 4.53 Expression of indicated genes as determined by RT-PCR at 7^{th} and 14^{th} day after treatment. ICG concentration was 0.5 μM. * indicates statistically significant difference ($p<0.05$) compared to the control group.

and mineralization is increased in all groups. Assays performed at later time points may reveal more definite conclusions.

ROS staining and DPBF solution assays showed the presence of singlet oxygen and ROS after laser irradiation. This amount of ROS did not cause any cell damage but it may have lead to osteoblastic behavioral changes such as increased ALP and mineralization.

Currently, there are not many studies using PS-mediated PBM to stimulate stem cell differentiation (the few ones are discussed in the previous sections.) IGC and infrared light is usually combined in PDT studies to destroy cancerous cells or microorganisms. This study indicates that if PS is applied with appropriate light dose can improve cellular processes but further investigations are needed to clearly demonstrate the effects.

4.9 Discussion of parameters affecting the results

Light therapies like PBM have been studied for many years. The first encouraging results motivated many researchers to perform studies to understand the cellular basis of PBM and figure out methods to use it clinics. However, photochemical and photobiological reactions caused by PBM depend on many parameters such as wavelength, power, energy density, irradiation mode and cell type as well as other cell culture conditions. Moreover, the differences in experimental protocols have made more and more difficult to compare the results. In order to identify PBM parameters responsible for biostimulatory or bioinhibitory effects detailed analysis is necessary. In the present study, the parameters except wavelength or cell type were kept constant in order to be able to compare the outcomes. Three of the basic parameters that may have affect the results are discussed below.

Effect of Culture Medium: The serum concentration is one of those parameters, which are thought to have an effect on PBM studies. Nutritional deprivation causes stressful conditions for cultured cells [86] The serum, FBS, has been used for decades in cell culture even though it contains many factors that are not controlled and which are variable. Using chemically defined, serum-free media may be an alternative but their ability to promote cell proliferation should be checked [87]. The studies can be divided into three according to serum concentrations in their growth medium: serum-free, low

serum (2 or 5%) and ideal (10 or 15%). Few studies have investigated PBM using serum-free medium. Zhang et al. irradiated primary myoblasts by He-Ne laser and found that irradiated myoblasts can survive and proliferate in the absence of serum [88]. Use of the superpulsed CO_2 laser at fluences of 2.4 and 4.7 J/cm^2 shortened population doubling times of keloid fibroblasts relative to that of controls when they were cultured in serum-free medium [89].

Cells grown under nutritional deficit (low serum) responds differently to PBM compared to cells in ideal serum concentrations. It is proposed that when the cells are fully functional, the therapeutic effect will not be observed. Fujihara et al. used cell medium with 5% FBS to cause stress and showed that osteoblast-like cells irradiated at 780 nm (3 J/cm^2) had an increased proliferation rate [90]. Oliveira et al. concluded that applying 1.5 J/cm^2 laser light to odontoblast-like cell MDPC-23 cultures which are maintained under nutritional deficit conditions (5% FBS) had a trend towards more favorable results [91]. Similarly, in another study human gingival fibroblasts cultured in nutritional deficiency (5% FBS) and irradiated at wavelengths 670, 780, 692 and 786 nm (2 J/cm^2) showed significantly higher cell growth than the control group [92].

On the other hand, the serum concentration may not be the only reason for a proliferative laser irradiation because there are studies that show that even if cells are grown in low-serum media, PBM did not improve cell viability of myoblasts [93]. Tabatabaei et al. did not assess the effect of nutritional deficit when they studied osteogenic differentiation of dental pulp stem cells [43]. Moreover, the FBS concentrations that will cause stress stimulus may be different for each cell type since the responses are different for the same low serum concentration [94]. The cellular response to PBM obtained in the present study may be weakened since the cells are initially maintained under serum-sufficient growth conditions. Same parameters used in this study may be performed in nutritional deficit medium in order to clarify the effect of serum concentration in the future.

Biphasic Response of light:

Another reason for conflicting results may be the biphasic response of light: if the

irradiation is too low there will be no response, if too high the response is inhibitory [95]. The optimal conditions for time and energy may have a biostimulatory response also. Therefore, there exist several controversial results in the literature not only on the PBM but also on PDT studies. However, negative results do not imply that PDT or PBM are ineffective in general: may be the combination of chosen parameters were not adequate.

Irradiation number:

It has been proposed that the stimulatory response may depend on the timing of irradiation and total dose of it. Multiple dose irradiations can cause cumulative effect and stimulatory action may be detected easily. The studies in which positive effect of PBM is observed, repeated the light irradiation several times over the entire course of the experiment. It should be noted that number of PBM applications and timing between them is likely to play a role. This may be one of the reasons of the absence of PBM effect in these studies especially for cell viability and proliferation. A slight increase in cell viability is observed in some groups in this study after a single irradiation. The increase may be more prominent if the cultures were subjected to multiple applications.

5. CONCLUSION

The first goal of the study was to investigate red and near infrared laser effects on osteoblast and on differentiation of ASCs into osteoblasts *in vitro* by qualitative and quantitative assays. The second part of the study focused on whether low level laser irradiation effects could be enhanced (if it exists) if it is mediated by photosensitizers.

The significant differences found in the studies are summarized in the Table 5.1 and 5.2.

This is a comparative study. 635 and 809 nm results can be used to compare the effect of wavelength on the same cell type. For osteoblasts, 809 nm can be used to induce cell viability within short time after irradiation (48h) and this effect may be increased by multiple exposures. On the other hand, 635 nm at 0.5 J/cm^2 may be preferred if ALP activity increase is needed. Exposure at both wavelengths decreased mineralization. The wavelength comparison on ASCs differentiation has revealed different outcomes: 809 nm irradiation increased mineralization and 635 nm increased viability at 24h after exposure at 0.5 J/cm^2 energy density

Table 5.1
Significant outcomes of the experiments on osteoblasts.

Osteoblasts								
	MTT	**Alamar**	**AO/PI**	**ALP activity**	**Mineralization**	**BGLAP expression**	**ALPL expression**	**COL1A expression**
635 nm	no effect	no effect	no effect	increase in $0.5J/cm^2$ group at 7^{th} day	decrease in all groups except for 1 J/cm^2 at 14^{th} day	decrease in all groups at 7^{th} day	decrease in $1J/cm^2$ group at 7^{th} day	increase in $2J/cm^2$ group at 14^{th} day
635nm+MB	decrease in all groups at 72h	no effect	no effect	increase in all laser groups at 7^{th} day	decrease in all groups at 7th and 14^{th} day	no effect	increase in 2 J/cm^2 group at 7^{th} day	increase in $2J/cm^2$ group at 14^{th} day
809 nm	increase in all groups at 48h	no effect	no effect	no effect	decrease in all groups at 14^{th} day	decrease in all groups at 7^{th} day and in 1 and $2J/cm^2$ at 14^{th} day	increase in all groups at 7^{th} day	no effect
809 nm+ICG	increase in 2 J/cm^2 group at 24h decrease in 1 and $2J/cm^2$ group at 72h	no effect	no effect	no effect	decrease in all groups at 7^{th} day increase in all groups at 14^{th} day	increase in $0.5J/cm^2$ group at 7^{th} day decrease in $1J/cm^2$ group at 7^{th} day	increase in ICG group at 7^{th} day decrease in $2J/cm^2$ group at 14^{th} day	increase in $1J/cm^2$ group at 14^{th} day

Table 5.2
Significant outcomes of the experiments on adipose-derived stem cells differentiated into osteoblasts.

Adipose –derived stem cells differentiated into osteoblasts								
	MTT	**Alamar**	**AO/PI**	**ALP activity**	**Mineralization**	**BGLAP expression**	**ALPL expression**	**COL1A expression**
635 nm	increase in 0.5J/cm^2 group at 24h	no effect	no effect	no effect	decrease in 2 J/cm^2 group at 7th day decrease in 1 J/cm^2 at 14th day increase in 2 J/cm^2 group at 7th day	decrease in 0.5J/cm^2 group at 14th day	no effect	decrease in 1 J/cm^2 group at 14th day
635nm+MB	no effect	no effect	no effect	increase in all groups at 14th day	increase in MB group at 7th day decrease in 0.5 and 1 J/cm^2 groups at 7th day decrease in 1 J/cm^2 at 14th day	no effect	increase in MB and MB+0.5 J/cm^2 groups at 7th day	no effect
809 nm	no effect	no effect	no effect	no effect	increase in all groups except for 2J/cm^2 at 7th day	no effect	decrease in 0.5 and 1 J/cm^2 group at 7th day	decrease in 0.5 and 1 J/cm^2 group at 7th day
809 nm+ICG	no effect	no effect	no effect	increase in 0.5 and 1 J/cm^2 groups at 7th day increase in all groups at 14th day	increase in all groups at 14th day	decrease in ICG group at 7th and 14th day	no effect	no effect

The effects at the same wavelength on different cell types can also be compared. 809 nm decreased mineralization of osteoblasts but increased it when they are differentiated from ASCs. ALP activity is mostly altered by the use of 635 nm in both cell types. Once more, it can be concluded that the PBM depends on cell type and wavelength choice.

The PS-mediated studies did not cause any evident synergistic effect, at least for the parameters chosen in this study. However, small alterations especially in gene expressions should be considered for PDT studies. These results may contribute to the low-dose PDT researches.

Although PBM and PDT have different purposes, both have potential clinical relevance, which should have studied carefully since inappropriate conditions may lead to undesirable results for both therapies.

As a result, the bottom line appears to be that PBM may have an effect on cells but this possible stimulation depends on several parameters that are not defined clearly yet. The first preliminary studies in literature on PBM showing increased cell number or proliferation seemed to be promising but well designed and detailed examinations on gene expression levels raise a question mark in minds about the efficiency of PBM. However, negative results do not imply that PBM are ineffective in general: may be the combination of chosen parameters was not adequate. The potential applications of PBM may be numerous but adequate and reliable evidence is necessary to recommend PBM for clinical use.

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