LOW LEVEL LASER THERAPY ON HUMAN ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS

by

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Babama…

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ABSTRACT

LOW LEVEL LASER THERAPY ON HUMAN ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS

Recent in vitro studies on cell cultures provided that low level lasers have various biostimulatory effects on several tissues. Biostimulation of tissues and cells is an important issue in Tissue Engineering Applications like regeneration from stem cells. Therapeutic potential of mesenchymal stem cells (MSCs) from bone marrow or umbilical cord are now being tested for many lethal and chronic disorders worldwide; however the clinical use of these MSCs has presented problems. Current results indicate that adipose tissue can be a novel and abundant source for adult MSCs. In this study, the effects of 650 nm and 635 nm diode laser on proliferatıon of human adipose tissue derived mesenchymal stem cells were examined. Two different dosages (1,6 J/cm2 and 3,2 J/cm2) were applied for both two wavelengths. 24 h after seeding, irradiation was started. Cells were exposed to laser irradiation for three consecutive days. Cells were counted at 5th, 9th, 14th and 21st days after seeding. It was observed that the extent of enhancement of the cell proliferation by 650 nm diode laser was significantly higher relative to control group at 14th day. 635 nm diode laser application results showed significantly higher growing enhancement relative to control group on 5th day.

Keywords: Low Level Laser Therapy, Biostimulation, Mesenchymal Stem Cell, Cell Proliferation.

ÖZET

İNSAN YAĞ DOKUSUNDAN ELDE EDİLEN MEZENKİMAL KÖK HÜCRELERDE DÜŞÜK DÜZEY LASER TERAPİSİ

Son dönemlerde hücre kültürleri ile yapılan in vitro çalışmalar, düşük düzey laserlerin farklı dokularda çeşitli biyo-uyarım etkilerinin olduğunu göstermiştir. Doku ve hücrelerin biyo-uyarımı, kök hücrelerden rejenerasyon gibi Doku Mühendisliği Uygulamaları'nda önemli bir konudur. Göbek kordonu ve kemik iliğinden elde edilen mezenkimal kök hücrelerin (MKH) birçok ölümcül ve kronik hastalık için tedavi potansiyelleri tüm dünyada denenmektedir; fakat bu hücrelerin klinik uygulamalarında problemler çıkmaktadır. Yakın zamanda yürütülen çalışma sonuçları yağ dokusunun yetişkin MKH'ler için yeni ve bol bir kaynak olabileceğini göstermektedir. Bu çalışmada, 650 nm ve 635 nm diyot laserlerin, insan yağ dokusundan elde edilen mezenkimal kök hücrelerin çoğalması üzerine etkisi araştırılmıştır. Her iki dalgaboyu için iki farklı doz (1,6 J/cm2 and 3,2 J/cm2) kullanılmıştır. Ekimden 24 s sonra radyasyon başlatılmıştır. Hücreler ard arda üç gün boyunca laser ışımasına maruz bırakılmıştır. Hücreler ekimden sonraki 5., 9., 14. ve 21. günlerde sayılmıştır. 650 nm laserin hücre çoğalmasındaki artışa etkisinin 14. günde kontrolden daha fazla olduğu gözlenmiştir. 635 nm laser uygulaması, 5. günde kontrole göre büyümede daha yüksek bir artış göstermiştir.

Anahtar Sözcükler: Düşük Düzey Laser Terapisi, Biyo-uyarım, Mezenkimal Kök Hücre, Hücre Çoğalması.

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LIST OF SYMBOLS

p-value	Probability value in *t*-test
Fcr-value	F critic value of ANOVA
°C	Degree Celsius
Cu	Cupper

LIST OF ABBREVIATIONS

LASER	Light Amplification by Stimulated Emission of Radiation
MSCs	Mesenchymal Stem Cells
ESCs	Embryonic Stem Cells
BMSCs	Bone Marrow Stem Cells
ADMCs	Adipose Tissue Derived Mesenchymal Stem Cells
LLLT	Low Level Laser Therapy
hADMSCs	Human Adipose Tissue Derived Mesenchymal Stem Cells
ASCs	Adult Stem Cells
IVF	In Vitro Fertilization
SSCs	Somatic Stem Cells
CD	Cell Differentiation
ICM	Inner Cell Mass
HFEA	Human Fertilization and Embryology Authority
HSCs	Hematopoietic Stem Cells
KDR	Kinase Insert Domain Containing Receptor
mdx	Dystrophin-Deficient Mice
lin–	Lineage –
CFU-AP	Colony Forming Unit - Alkaline Phosphatase
VEGF	Vascular Endothelial Growth Factor
HGF	Hepatocyte Growth Factor
SVF	Stroma Vascular Fraction
PBM	Photobiomodulation
ATP	Adenosine Triphosphate
cyt	Cytochrome
IR	Infra-Red
Redox	Oxidation-Reduction Reaction
GaAlAs	Gallium-Aluminum-Arsenium
RANK	Receptor Activator of Nuclear Factor-Kappa-B
OP	Osteopontin

BSP	Bone Sialoprotein
He-Ne	Hellium-Neon
ALP	Alkaline Phosphatase
Ga-As	Gallium Arsenide
GM-CFC	Granulocyte–Macrophage Colony Forming Cells
SDF-1	Stromal Cell-Derived Factor-1 Alpha
FDCP-mix	Factor-Dependent Cell Paterson mix
SEM	Scanning Electron Microscopy
EDS	Electron Dispersive Spectrometry
CSCs	Cardiac Stem Cells
ANOVA	Analysis of Variance
PBS	Phosphate-Buffered Saline
DMEM	Dulbecco's Modified Eagle's Minimal Essential Medium
FBS	Fetal Bovine Serum
L-Glutamine	Glutamine with Amino Group on Left
EDTA	Ethylenediamine Tetraacetic Acid
DMSO	Dimethyl Sulfoxide
SD	Standard Deviations
St. Sign.	Statistically Significant
DNA	Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
mW	Milliwatt
Hz	Hertz
C	Control Group
L1	Laser Group 1
L2	Laser Group 2
J	Joule
n	Sample Size
ml	Milliliter
µl	Microliter
mg	Milligram
nm	Nanometer

1. INTRODUCTION

1.1 Motivation

Stem cells differ from other kinds of cells in the body. All stem cells -regardless of their source- have three general properties: being unspecialized, giving capable of dividing and renewing themselves for long periods and rising to specialized cell types such as a muscle cell, a red blood cell or a brain cell. With the remarkable potential to develop into many different cell types, they serve as a sort of repair system for the body.

Therapeutic potential of stem cells from bone marrow or umbilical cord are now being tested for many lethal and chronic disorders worldwide. However, the clinical uses of bone marrow derived and umbilical cord derived mesenchymal stem cells (MSCs) have presented problems including pain, morbidity, and low cell number upon harvest. On the other hand, although the potential of embryonic stem cells (ESCs) is enormous, many ethical and political issues accompany their use. This has led many researchers to investigate alternative sources for MSCs.

Adipose tissue is derived from the embryonic mesenchyme and contains a supportive stroma structure which is similar to the bone marrow MSCs (BMSCs) and researches have proved that adipose tissue derived MSCs (ADMSCs) can be induced into multiple lineages in specific culture systems similar to bone marrow and umbilical cord MSCs. Moreover, ADMSCs are much more abundant, easier to obtain, there is much less cell heterogeneity and also adipose tissue has a large number of cells. They are also capable of expressing multiple growth factors. Therefore, it is believed that ADMSCs represent an ideal substitute for other stem cells used in Cell-based Tissue Engineering, Bioengineering and Regenerative Medicine Applications.

Scientists in many laboratories are trying to find ways to grow stem cells and manipulate them to generate specific cell types *in vitro.* And recent *in vitro* studies suggest that in response to suitable microenvironments and/or stimuli stem cells can be expanded

and terminally differentiated into other specific cell types. One such inducing factor might be low level laser therapy (LLLT) which has been shown to have a variety of biostimulatory effects, such as promotion of wound healing, cell proliferation and regeneration.

LLLT application can also be beneficial to enhance the proliferation of ADMSCs. Importantly, in an injured or an ischemic organ like the heart, brain, etc. the time of implantation post-ischemia/injury may be crucial to achieve maximum beneficial clinical effect. Furthermore, since LLLT has been shown to induce a cytoprotective effect on cells the laser-irradiated cells may have a better long-term survival post-implantation and can serve better as stem cells for production of growth factors in the host organ or participate in the regenerative process.

Today's scientific interest in Tissue Engineering for organ transplantations and regeneration from stem cells, combined with recent observations on biostimulation of tissues and cells by laser radiation, stands as a strong motivation for the present work.

A broad literature exists on the use of LLLT in various experimental biological models. However there have been a few studies for LLLT on stem cells and although positive results of these studies, there have been no sufficient work for optimum parameters of LLLT for different stem cell types yet and need further investigations. Moreover, as far as it is concerned, there has been no work on LLLT for ADMSCs.

The major aim of this experimental study is to assess the effects of 650 nm diode laser and 635 nm diode laser irradiation on human adipose tissue derived mesenchymal stem cells (hADMSCs) *in vitro*.

1.2 Outline

In Chapter 2, general properties of stem cells, major types of stem cells with their specific properties and various application areas are explained to clear the importance of

stem cells. LLLT mechanism hypothesizes are viewed to be able to interpret the results of this study. A broad review of recent literature is given for LLLT applications on different cell types and also stem cells that used to choose optimum parameters for this application.

Chapter 3 clearly demonstrates the importance of this study and explains how every step of the study was planned and what their specific reasons are.

Chapter 4 gives detailed information about materials and methods used in this study such as cell thawing, cultivation, laser irradiation parameters, experimental groups, cell counting and statistical analysis.

Results about the effects of low level laser irradiation in two different wavelengths are shown in Chapter 5. Effects of different energy dosages and comparisons of groups for various time intervals were also shown to be able to discuss the results more clearly.

In Chapter 6, a brief discussion of overall results and contribution of this study for further scientific works can be found.

2. BACKGROUND

2.1 Stem Cells

2.1.1 General Properties of Stem Cells

Research on stem cells is advancing knowledge about how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. This promising area of science is also leading scientists to investigate the possibility of cell-based therapies to treat disease, which is often referred to as regenerative medicine [1]. A stem cell is traditionally defined as a cell that is capable of self-renewing and differentiating into an unlimited number of different cell types [2, 3] such as the beating cells of the heart muscle [4, 5] or the insulin-producing cells of the pancreas [6].

Scientists primarily work with two kinds of stem cells from animals and humans: ESCs and adult stem cells (ASCs), which have different functions and characteristics. Researchers discovered ways to obtain or derive stem cells from early mouse embryos more than 20 years ago. Many years of detailed study of the biology of mouse stem cells led to the discovery, in 1998 (James Thomson et al), of how to isolate stem cells from human embryos and grow the cells in the laboratory. The embryos used in these studies were created for infertility purposes through *in vitro* fertilization (IVF) procedures and when they were no longer needed for that purpose, they were donated for research with the informed consent of the donor.

Stem cells are important for living organisms for many reasons. They are crucial for the cell homeostasis of the body, primarily because their main role is to compensate for the natural loss of cells fulfilling specialized functions [7]. In the 3 to 5 day-old embryo, called a blastocyst**,** stem cells in developing tissues give rise to the multiple specialized cell types that make up the heart, lung, skin, and other tissues. In some adult tissues, such as bone

marrow, muscle and brain, discrete populations of ASCs generate replacements for cells that are lost through injury or disease [1].

The differentiation potential or plasticity of a stem cell is its ability to produce progeny that express various mature phenotypes. The maintenance of many tissues and organs is achieved by tissue-specific stem cells. In general, stem cells divide very rarely, but in the presence of an appropriate stimulus, for example an increasing demand for cells, they proliferate and differentiate [8].

It has been hypothesized by scientists that stem cells may, at some point in the future, become the basis for treating diseases such as Parkinson's disease, diabetes, Alzheimer's disease, cancer and heart disease. Today, donated organs and tissues are often used to replace those that are diseased or destroyed. Unfortunately, the number of people needing transplants far exceeds the number of organs available. Stem cells offer the potential for supplying cells and tissues, which can be used to treat these various diseases [9]. In order to develop such treatments, scientists are intensively studying the fundamental properties of stem cells, which include determining precisely how stem cells remain unspecialized and self renewing for many years and identifying the signals that cause stem cells to become specialized cells.

2.1.2 Types of Stem Cells

All human beings start their lives from a single cell, called the zygote, which is formed after fertilization. The zygote divides and forms two cells; each of those cells divides again, and so on. Soon, about five days after conception, there is hollow ball of about 150 cells called the blastocyst. The blastocyst contains two types of cells, the trophoblast and the inner cell mass. ESCs are the cells that make up the inner cell mass. As ESCs can form all cell types in an adult, they are referred to as pluripotent stem cells.

Stem cells can be found in very small numbers in various tissues in the adult body. For instance, BMSCs are found in the marrow of the bone and they give rise to all

specialized blood cell types. ASCs are typically programmed to form different cell types of their own tissue; they are called multipotent stem cells. ASCs have not yet been identified in all vital organs. In some tissues like the brain, although stem cells exist, they are not very active, and thus do not readily respond to cell injury or damage. Scientists are now also exploring ways in which they can induce the stem cells already present to grow and make the right cell types to replace the damaged ones.

Stem cells can also be obtained from sources like the umbilical cord of a newborn baby [10, 11]. This is an accessible source of stem cells, compared to adult tissues like the brain and bone marrow. Although scientists can grow these cells in culture dishes, they can do so only for a limited time. Recently, scientists have discovered the existence of stem cells in amniotic fluid -surrounds an unborn baby- [12 – 14] and stem cells in placenta [13, 15] and these cells may also have the potential to form multiple cell types. Research to characterize and study these cells is very promising but at a very early stage.

They are hard to identify all cell types of the body. Their main physiologic characteristic, their ability to divide indefinitely, does not represent a useful marker for identification. Currently, the main cell surface markers for isolation of somatic stem cells (SSCs) come from the haematopoietic lineage such as the cell differentiation (CD) markers CD34 and CD133. Whether there are unique cell surface markers for stem cells is still an area of debate as those found so far can appear, disappear and reappear [16]. There are stem cell markers for ESCs; however these appear to have different expression between the various human ESCs lines [17].

The shared properties of stem cells, as well as their distinguishing features, have fascinated and puzzled the research communities for more than three decades. The need to understand the basis of multiple lineages potential arises from both a practical aspiration to use stem cells effectively for medical research and from the point of view of elaborating in detail how cell-fate decisions are made and memorized [18].

Stem cells are classically defined as cells that can both self-renew and generate progeny that are capable of following more than a single differentiation pathway, unlikely any specific adult cell. In the case of ESCs, which are pluripotent, the range of lineage options available to each cell is large, theoretically representing an unlimited source of every type of tissue that is found in the adult animal to engineer diseased tissues to regain normal function [18 – 20].

The last twenty five years have seen the development of techniques to isolate ESCs. Mouse ESCs were first isolated and grown in the UK in 1981 [21]. Since then, hESCs have been isolated and grown in the USA and Israel [22, 23]. Now, many laboratories world-wide are isolating hESCs.

hESCs have unique proliferative and differentiation characteristics which makes them a valuable cell source for cell transplantation and Tissue Engineering. These cells are derived from fertilized embryos less than a week old. Using blastocysts obtained from donated, surplus embryos produced by IVF, a group of UW-Madison developmental biologists led by James Thomson established five independent stem cell lines in November 1998. This was the first time hESCs had been successfully isolated and cultured. The embryos used in the work were originally produced to treat infertility and were donated specially for this project with the informed consent of donor couples who no longer wanted the embryos for implantation [19]. In virtually, in every IVF clinic in the world, surplus embryos are discarded if they are not donated to help other infertile couples. Alternatively, spare embryos, whether healthy, cryopreserved or the ones carrying a genetic abnormality may be donated to research laboratories.

Donated embryos are cultivated *in vitro* to day 5-6 when hatching from the zona pelucida should occur. Hatched blastocysts consist of an outer layer of cells, the trophectoderm, which will go on to form the extra-embryonic structures such as the placenta, and the inner cell mass (ICM). The ICM is isolated by immunosurgery or by mechanical separation and the cells of the ICM grown *in vitro* tissue culture conditions [22]. These cells constitute the ESCs.

Identifying ESCs, requires specific markers. The main antigen markers for hESCs are Oct 4 and some stage-specific embryonic antigens (SSEA- 3 and 4, TRA-1-60 and 81) which vary between those found on the mouse ESCs and those found on human ESCs [24]. AP and high levels of telomerase are expressed by hESCs [22, 25].

As long as the ESCs in culture are grown under certain conditions, they can remain undifferentiated (unspecialized); however, if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell types. Although spontaneous differentiation is a good indication that a culture of ESCs is healthy, it is not an efficient way to produce cultures of specific cell types. Therefore, to generate cultures of specific types of differentiated cells scientists try to control the differentiation of ESCs [26].

Since hESCs were first derived, there have been many reports describing their ability to differentiate into a variety of specific cell types, including neurons (Carpenter et al, 2001; Reubinoff et al, 2000), cardiomyocytes (Xu et al, 2002), endothelial cells (Levenberg et al, 2002), islets (Assday et al, 2001), trophoblasts (Xu et al, 2002), hematopoietic cells (Kaufman et al, 2001) and other cell types. The tissue-specific cell types derived from hESCs may have therapeutic potential for the treatment of Parkinson's disease, spinal cord injury, heart disease, diabetes and other degenerative conditions.

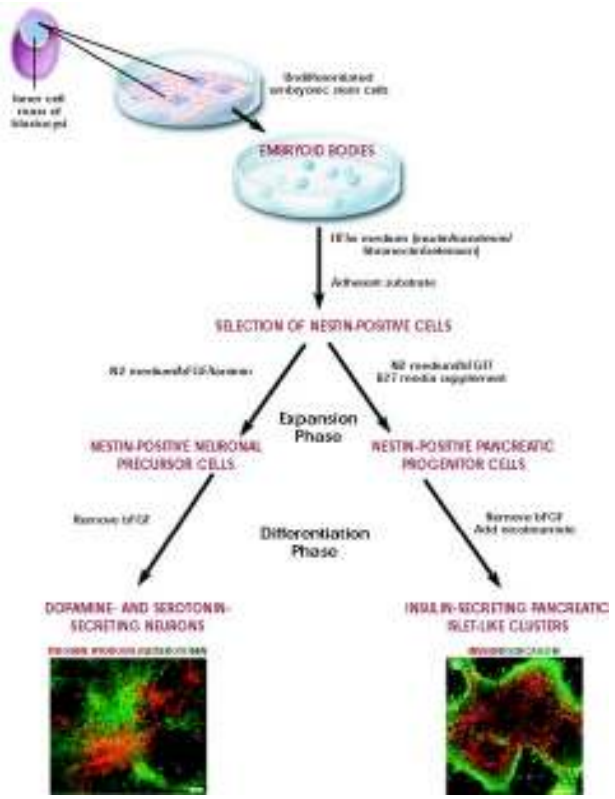

Figure 2.1 Directed differentiation of mouse embryonic stem cells [32].

To realize the potential of these cells, reproducible and reliable production of hESCs under controlled conditions is critical. In order to develop a convenient, consistent procedure to produce cells at large scale with minimal animal-derived components, it would be beneficial to utilize a culture medium that is defined, contains only human derived or recombinant proteins, and does not require conditioning with feeder cells [27]. The recent developments of humanized, feeder-free culture systems to derive and culture human ESC, methods to genetically modify the cells, and strategies to derive highly enriched populations of functional differentiated cells of a specific type are encouraging. It is anticipated that these achievements will set the stage for further developments that may eventually allow using the potential of hESCs for cell and gene therapy [28].

Research and clinical use of ESCs is a global process and hESCs are valuable for biomedicine; but differing cultural, political, legal, and religious perspectives are potential barriers to international collaboration in this fledgling field [29]. Throughout the world, the legislature of each country regulates the use of embryos from IVF. In Europe, there is

variation of practice and legislature between each Member State. The USA also has variation in practice and legislature from State to State with a Federal embargo on the derivation of new ESCs lines currently.

In UK an independent body, the Human Fertilization and Embryology Authority (HFEA) was created in 1990. This body licenses all reproductive medicine and human embryo research in the UK. Originally the HFEA was set up to regulate reproductive medicine, but later acts of Parliament have now allowed embryonic research [30].

Turkey is among several countries in which no specific regulations and guidelines have so far been defined by legal or governmental institutions for hESCs research. Necati Findikli reported the first known derivation of hESCs from donated blastocyst-stage embryos in Turkey in 2005 (Reproductive Medicine Online, 2005). At September 2005, with the guideline numbered 19.09.2005 – 17972, hESCs researches were stopped by The Ministry of Health of Turkey until specific regulations are defined [31].

An adult stem cell is an undifferentiated cell found among differentiated cells in a tissue or organ, can renew itself and can differentiate to yield the major specialized cell types of the tissue. The primary roles of ASCs in a living organism are to maintain and repair the tissue in which they are found. The term “adult” is used to distinguish any multipotent cell from ESCs. Some scientists now use the term SSCs instead of adult stem cell and some others even use the term “fetal” stem cells for ASCs, which are derived from fetal tissue prior to birth. Unlike ESCs, which are defined by their origin (the inner cell mass of the blastocyst), the origin of ASCs in mature tissues is unknown [26].

Based on historical experiments in different animal models, an adult stem cell was first defined in the hematopoietic system; McCulloch and Till illustrated the presence of self-renewing cells in mouse bone marrow, in 1963. Previously, most of the published data with respect to the differentiation of precursor cells used already predetermined progenitor cells, derived from the organ of origin, e.g., allowing chondroblasts to differentiate into cartilage-like tissue. Due to the limited availability of tissue stem cells, there is a growing

scientific and commercial interest in the potential of ASCs derived from other sources, like the bone marrow, umbilical cord, or peripheral blood [32].

The potential of hematopoietic progenitor cells is yet best understood among all ASCs, since they have been isolated and investigated first after experiencing the effect of nuclear bombs in Hiroshima and Nagasaki. Today, adult hematopoietic stem cells (HSCs) are routinely isolated from the bone marrow, umbilical cord, and the peripheral blood based on the expression of the CD34 and/or CD133 antigens [35]. It also has been shown that it is possible to achieve full lympho-hematopoietic reconstitution with CD34– mesenchymal-type cells (CD34–) [36, 37].

Examples of the versatile nature of multipotent ASCs have been published in many experiments to date. They include the apparent "trans-differentiation" of BMSCs into myoblasts [38], endothelium [39–42], liver, biliary duct [43–45], lung, gut [46] or even neural tissue [47, 48]. Some authors claim that the injection of BMSCs into the heart may lead to the differentiation of progenitor cells into cells with a cardiomyoblast-like phenotype [49]; although these results are currently an issue of controversy. Other authors showed that CD34– cells from the bone marrow contain precursor cells of hepatic tissue ("hepatic oval cells") [50]. There have been further examples of cells with stem cell potential, which were defined by the expression of angiogenic receptor proteins, e.g., kinase insert domain containing receptor (KDR) [51]. It was further shown that HSCs also can differentiate *in vitro* into neural progenitors, myogenic tissue progenitors [52, 53] and that dystrophin expression can be restored after hematopoietic stem cell transplantation in dystrophin-deficient mice (mdx) [54]. All those experiments led to the discussion on plasticity of stem cells, suggesting that ASCs from different sources and of almost any tissue phenotype can "trans-differentiate" into other tissue progenitors depending on the appropriate conditions or the adequate lesions [55, 56].

The isolation of ASCs at sufficient purity and quantity remains difficult [26]. Thus far, no definitive markers have been identified, which would allow a positive selection for ASCs. In general, stem cells are defined by not expressing most of the tissue-specific antigens (lin–). The fact that they have to undergo self-renewal by definition does not help

in the attempt to isolate enough cells with the characteristics of stem cells. However, self-renewal and self-maintenance are one of the most important features of stem cells, since they have to maintain a steady-state of organ function after injury or loss of function. ASCs can not be defined by a particular location within an organism (niche), because they are dispersed throughout almost any tissue in a mature organism at very different frequencies and states of activity.

They also seem to behave differently depending on their local environment. The usual environment includes the peripheral blood, which has been shown to be an easily accessible substrate for the isolation of mesodermal stem cells [57, 58]; also, the yield of stem cells is smaller than from other sources like the bone marrow are umbilical cord blood. The difficulties to isolate sufficient numbers of multipotent stem cells also is due to the mixture of cells, even among a CD34_ apheresis product, which usually contains endothelial cells, mature immune cells, and other monocytes. Therefore, it became desirable to generate well-characterized cell lines of consistent quality, at least to proof the principles of the concept [26].

Scientists do not agree on the criteria that should be used to identify and test ASCs. However, they often use one or more of the following three methods: (1) labeling the cells in a living tissue with molecular markers and then determining the specialized cell types they generate; (2) removing the cells from a living animal, labeling them in cell culture, and transplanting them back into another animal to determine whether the cells repopulate their tissue of origin; and (3) isolating the cells, growing them in cell culture, and manipulating them, often by adding growth factors or introducing new genes, to determine what differentiated cells types they can become [33].

ASCs and stem cell-derived tissues can be useful therapeutically in replacing cells and even tissues in humans. Contrary to the impression created by advocates of destructive hESCs research, the biomedical potential of ESCs remains entirely speculative. ESCs have never been successfully used in clinical applications with human patients. By contrast,

ASCs already have been used in a variety of human clinical trials and applications with considerable success [59].

Such treatments include applications for various types of cancer, including but not limited to: brain tumors [60-62], retinoblastoma [63-64], ovarian cancer [65-66], various solid tumors [67-71], testicular cancer [72-73], multiple myeloma and leukemias [74-78], breast cancer [79-82], neuroblastoma [83], non-Hodgkin's lymphoma [84-86] and renal cell carcinoma [87-88]. ASCs have also been used in treatment of autoimmune diseases such as multiple sclerosis, systemic lupus, rheumatoid arthritis, and juvenile rheumatoid arthritis [89-91], stroke [92], immunodeficiencies and anemias [93-96] and cartilage and bone diseases [97-98]. ASCs have been used to regenerate corneas, restoring sight to previously blind patients [99-102] and also to combat blood and liver diseases [103-104]. Recently, the positive results from the first successful human trials of ASCs to treat cardiac damage were published [105-106]. Simply stated, ASCs are already being used in a wide array of human clinical trials, with many therapeutic applications having moved well beyond the experimental stage.

The Guidelines asserted that ASCs are likely to be ineffective at combating genetic diseases because the patient's own stem cells would likely contain the same genetic error, making cells from the patient inappropriate for transplantation. However, evidence from clinical studies to date belies this assertion. The first successful human gene therapy used ASCs to cure severe combined immunodeficiency syndrome [108]. Not only can genetic error be remedied while ASCs are in culture, but in many cases the correction of the genetic defect may not be necessary to affect a cure with ASCs. For instance, patients with systemic lupus have been treated with their own adult BMSCs which repaired organ damage that was previously considered permanent. This repair occurred without correcting the genetic defect present in the bone marrow cells [109].

The multilineage potential of ESCs and ASCs from certain sources has been characterized extensively. Although ESCs potential is enormous, many ethical and political issues accompany their use. Therefore, ASCs from the bone marrow stroma have been proposed as an alternative source. Originally, identified as a source of osteoprogenitor

cells, BMSCs differentiate into adipocytes, chondrocytes, osteoblasts and myoblasts *in vitro* (Hauner et al, 1987; Grigoradis et al, 1988; Wakitani et al, 1995; Ferrari et al, 1998; Johnstone et al, 1998; Pittenger et al, 1999) and undergo differentiation *in vivo* (Benayahu et al, 1989; Bruder et al, 1998), making these stem cells promising candidates for mesodermal defect repair and disease management. However, the clinical use of BMSCs has presented problems including pain, morbidity, and low cell number upon harvest. This has led many researchers to investigate alternate sources for MSCs [112].

Adipose tissue is derived from the embryonic mesenchyme and contains a supportive stroma structure which is similar to the BMSCs. And also; researches have proved that ADMSCs can be induced into multiple lineages in specific culture systems similar to BMSCs. [110-112]. Recently, it has been reported that hADMSCs can be induced to express genes and protein markers associated with the chondrocyte, adipocyte, osteoblast, endothlium and myocyte [112-115].

Compared with BMSCs, ADMSCs are much more abundant and easier to obtain, carrying relatively lower donor site morbidity [116]. There is much less cell heterogeneity in ADMSCs than in BMSCs, which is caused by the mixture of HSCs and MSCs [110, 117-118]. And also adipose tissue has a large number of cells with CFU-F ability (colony-forming units-fibroblasts) or colonies expressing alkaline phosphatase (CFU-AP) [142]. They are also capable of expressing multiple growth factors, including vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [143]. Therefore, it is believed the ADMSCs represent an ideal substitute for BMSCs used in Cell-based Tissue-Engineering Applications.

After isolation of the stromal vascular fraction (SVF) from the adipose tissue, ADMSCs can be expanded in culture towards a homogeneous cell population for extended periods [112, 113]. The cultured cells show low levels of cell senescence and importantly, maintain their multipotency towards the mesenchymal lineage [113,120, 141]. Cultured ADMSCs have the capacity to differentiate into adipocytes [112, 113]; chondrocytes [112, 113, 121, 122]; osteoblasts [123, 124]; myocytes [112]; neuronal cells [125, 126] and cardiomyocytes [127]. These cells have recently been used to heal critical-size skeletal

defects in mice *in vivo* (Cowan et al, 2004). During the past several years, there has been growing interest in the applicability of ADMSCs for Stem Cell Technology and future cell-based therapies [116, 128].

Adipose tissue is available in large quantities and is easily obtained via surgical procedures. The three most commonly used procedures are resection, tumescent or conventional liposuction and ultrasound-assisted liposuction, which mostly aim at the improvement of the body-contour [129-131]. With tumescent liposuction, the donor material is infiltrated with a mixture of adrenaline and lidocaine, causing vasoconstriction and anesthesia. Ultrasound- assisted liposuction, introduced by Scuderi et al in 1987 [132], employs the use of ultrasonic energy to allow selective destruction of subcutaneous adipose tissue [133-135]. All three surgical procedures result in minimal patient discomfort and little donor site morbidity.

The potentially detrimental effect of the surgical procedures on functional characteristics of ADMSCs investigated by Varma et al, and they showed the yield and growth characteristics of are affected by the type of surgical procedure used for adipose tissue harvesting. Lower ADMSCs yields and growth capacities were observed with ultrasound-assisted liposuction material, and therefore resection and tumescent liposuction seem to be preferable rather than ultrasound-assisted liposuction for Tissue-Engineering purposes [128].

The recent surge of interest in adipose tissue as a source of adult multipotent stem cells also generated encouraging results for their use in regenerative medicine [136,137]. Adipose tissue is considered as an abundant and practical source of stromal precursor cells with great potential for soft-tissue repair and reconstruction [139, 140].

Further studies are necessary before ADMSCs can be used clinically. In particular, investigators need to demonstrate the safety and efficacy in animal models, either alone or in combination with biomaterial scaffolds. In addition, manufacturing and quality assurance issues need to be addressed. Although challenges remain, it is clear that

ADMSCs hold significant promise for future applications [144] and it is needed to develop new techniques to be able to ease *in vitro* studies of these cells.

2.2 Low Level Laser Therapy

In 1967, a few years after the first working laser was invented, Endre Mester in Semmelweis University (Budapest, Hungary), wanted to test if laser radiation might cause cancer in mice [170]. He shaved the dorsal hair of the mices, divided them into two groups and gave a laser treatment with a low powered ruby laser (694-nm) to one group. They did not get cancer and to his surprise the hair on the treated group grew back more quickly than the untreated group. This was the first demonstration of "laser biostimulation". Currently, also known low-level laser (or light) therapy (LLLT) is practiced as part of physical therapy in many parts of the world. In particular, Karu and coworkers have extensively published their results on that subject.

LLLT is defined as the application of red and near infrared light over injuries or wounds to improve soft tissue healing and relieve both acute and chronic pain and also at recent studies over tissue/cell cultures *in vitro* to achieve enhanced cell proliferation and differentiation for the usage of Tissue Engineering Applications. Low-level therapy uses cold (subthermal) laser light energy to direct bio-stimulative light energy to the body's cells without injuring or damaging them. Low-level laser light is compressed light of a wavelength from the cold, red part of the spectrum of electromagnetic radiation. It is different from natural light in that it is one precise color; coherent (it travels in a straight line), monochromatic (a single wavelength) and polarized (it concentrates its beam in a defined location or spot).

These properties allow laser light to penetrate the surface of the skin with no heating effect, no damage to the skin and no known side effects. The power range of low level laser irradiation is wide and changes according to the application. LLLT includes wavelengths between 500 and 1100 nm [146].

LLLT has been investigated and used clinically for over 30 years, and the worldwide interest in LLLT is illustrated by its use in more than 85 institutions in over 37 countries [147]. Reports of LLLT applied to soft tissues *in vitro* and *in vivo* suggest stimulation of specific metabolic processes, although low doses of LLLT are stimulatory, high doses may be suppressive [148]. For stimulative effects, there is a narrow and well-defined range of parameters of light, while the inhibitive fluence and intensity ranges are not so exactly determined and are much broader [149].

In LLLT, the question is no longer whether light has biological effects; but rather how energy from therapeutic lasers works at the cellular and organism levels and what are the optimal light parameters for different uses of these light sources. Parameters such as wavelength, fluence, and intensity play the most important roles in both the stimulation and inhibition of cellular metabolism [150].

Irradiation of cells at certain wavelengths can activate some of the native components. In this way, specific biochemical reactions as well as whole cellular metabolism can be altered. This type of reaction is believed to form the basis for low-power laser effects [151-153]. It should be noted that light therapy methods based on photochemical conversion of photo-absorbing molecules are not laser-specific methods. Conventional light sources generating the appropriate wavelength can also be used. Laser sources are just handy tools providing many practical advantages (e.g., efficient fiber-optic coupling to irradiate interior body parts, high monochromaticity and easy wavelength tunability, simplicity of use and electrical safety in the case of semiconductor lasers) [154].

2.2.1 Mechanism of Low Level Laser Therapy

There are numerous reviews previously published on various aspects of low-power laser effects and their mechanism, quantitative laws of visible-light action on cells photobiological fundamentals and molecular mechanisms [151, 152, 154]. Photobiomodulation (PBM) is a modulation effect of low intensity monochromatic light or LLLT on biosystems [188]. As Karu has pointed out [187], there is no effect of LLLT on

the cell which redox potential is so that the cell normally functions, and the lower the redox potential of a cell comparing with the normal redox potential, the stronger the effect of LLLT.

A photobiological reaction involves the absorption of a specific wavelength of light by the functioning photoreceptor (photoacceptor) molecule. The photoacceptors take part in a metabolic reaction in a cell which is not connected with a light response. After absorbing the light of the wavelength used for irradiation, this molecule assumes an electronically excited state from which primary molecular processes can lead to a measurable biological effect in certain circumstances. To work as a photoacceptor taking part in photobioregulation, this molecule must be part of a key structure that can regulate a metabolic pathway. Redox chains are an example of this type of key structure which fits these requirements [154].

Several lines of evidence show that mitochondria are sensitive to irradiation with monochromatic visible light. It was shown that the illumination of isolated rat liver mitochondria increased the adenosine triphosphate (ATP) synthesis and consumption of O_2 [158-161]. Irradiation with light at wavelengths of 415 [159], 602 [161], 632.8 [160], 650 [158] and 725 nm [158] enhanced the ATP synthesis. Light at wavelengths of 477, 5 [158] and 554 nm [159] did not affect the rate of the process. Oxygen consumption was activated under illumination with light at 365 and 436 nm, but not at 313, 546 and 577 nm [161]. It is also believed that mitochondria are the primary targets when the whole cells are irradiated with light at 630 [162], 632.8 [163-165] or 820 nm [166].

PBM on mitochondria was widely studied in the isolated form, in cells and *in vivo* respectively. There are also a few papers studying PBM on endoplasmic reticulum [193-195] and on Golgi apparatus [192, 193, 195]. The cross-talking between mitochondria, endoplasmic reticulum and microtubules has been found [191]. There should be the cross-talking between cellular organelles so that Schenk et al [195] and Pourzarandian et al [193] have found the simultaneous change of mitochondria, endoplasmic reticulum and Golgi apparatus, respectively. Therefore, the cellular organelles and their cross-talkings might also mediate cellular PBM [196].

The question is which molecule in a mitochondrion is responsible for the effects mentioned above. When considering the cellular effects, Karu et al suggested that this question can be answered with the aid of action spectra [154]. This is a graph representing biological photo-response as a function of wavelength, wave number, frequency, or photon energy and should resemble the absorption spectrum of the photoacceptor molecule. It is known that within certain limits, an action spectrum follows the absorption spectrum of the photoacceptor molecule.

On the other hand, the action spectrum is insufficient to distinguish between potential photoacceptor pigments with very similar absorption spectra. Moreover, the absorption spectrum for the photoacceptor pigment may depend strongly on its environment; however this environment remains unknown as long as the photoacceptor pigment itself is unknown. Because of these inadequacies of the photoacceptor pigment, some other criteria for identification like action spectra for the DNA and RNA synthesis rate suggested by Karu et al.

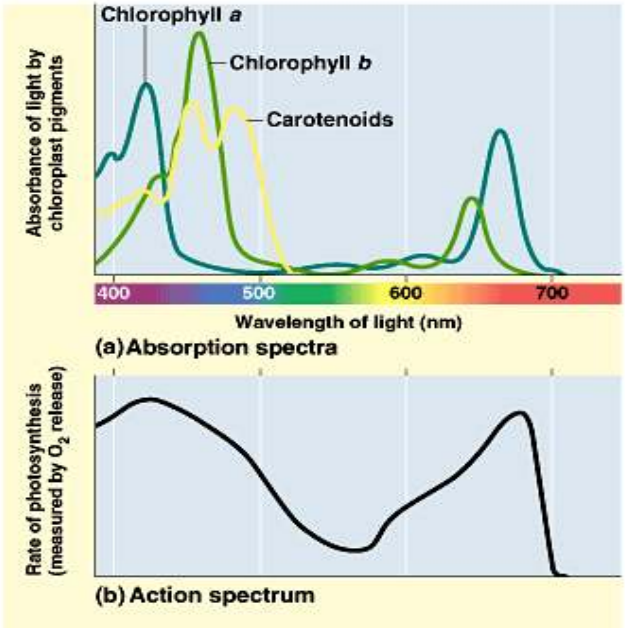

Figure 2.2 Action and absorption spectras [168].

It is known that the action spectrum is roughly the same shape as the absorption spectrum of the photoacceptor. Therefore, the bands in the action spectra were identified by analogy with the metal-ligand system absorption spectra characteristic of this spectral range. The regions 400-450 and 620-680 nm are characterized by the bands pertaining to complexes with charge transfer in a metal-ligand system, and within 760-830 nm, these are d-d transitions in metals. The region 400-420 nm is typical of π-π transitions in a porphyrin ring [154].

Comparative analysis of spectral data for transition metals and their complexes and biomolecules participating in the regulation of cellular metabolism allows suggesting that multinuclear enzymes containing Cu (II) may be participating. The action spectrum may be related to the cytochrome c oxidase [154]. The fact that the photoacceptors are components of the respiratory chain was considered earlier. Cytochrome c oxidase (or cyt a/a_3) is the terminal enzyme of the respiratory chain in eukaryotic cells, which mediates the transfer of electrons from cyt c to molecular oxygen. Ferrocytochrome c is oxidized, dioxygen is reduced, and protons are pumped vectorially from the mitochondrial matrix to the cytosol. Free energy resulting from this redox chemistry is converted into an electrochemical potential across the inner membrane of the mitochondrion, which ultimately drives the production of ATP. Accordingly, cytochrome c oxidase plays a central role in the bioenergetics of the cell and the terminal respiratory chain oxidases in eukaryotic cells (cytochrome c oxidase) are believed to be photoacceptor molecules for red to near-IR radiation.

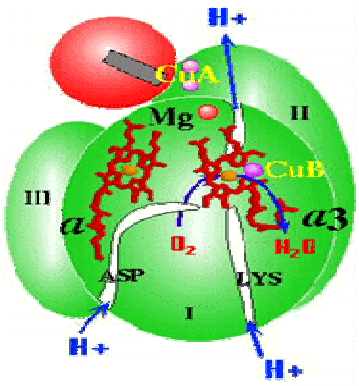

Figure 2.3 Model of cytochrome c oxidase (green) showing its three subunits (I-III) and its interaction with cytochrome c (red). Heme groups (a and a3) are depicted in red, CuA and CuB centers in purple [157].

The other important consideration involves the optical properties of tissue. Both the absorption and scattering of light in tissue are wavelength dependent (both much higher in the blue region of the spectrum than the red) and the principle tissue chromophore (hemoglobin) has high absorption bands at wavelengths shorter than 600-nm. For these reasons there is a so-called “optical window” in tissue covering the red and near-infrared wavelengths, where the effective tissue penetration of light is maximized (Figure 3). Therefore; although blue, green and yellow light may have significant effects on cells growing in optically transparent culture medium, the use of LLLT in animals and patients almost exclusively involves red and near-infrared light (600-950-nm) [171].

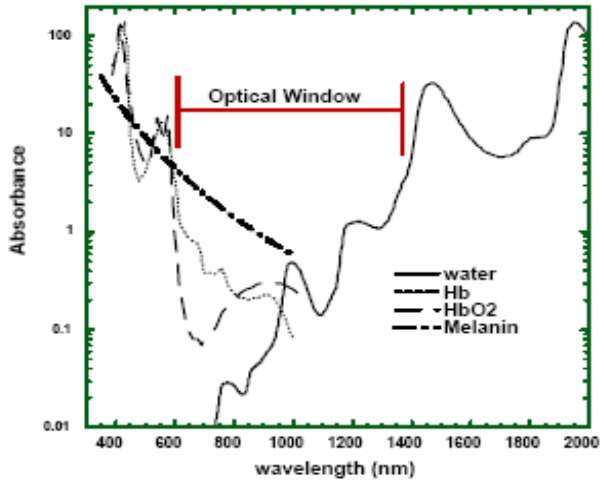

Figure 2.4 Optical window in tissue due to reduced absorption of red and near-infra-red wavelengths (600-1200 nm) by tissue chromophores [171].

There is certainly more than one reaction involved in the primary mechanisms of low-level laser effects. The four types of possible reactions are summarized in Figure 4. There are no grounds to believe that only one of these processes occurs when a cell is irradiated. However, recent experimental results of redox absorbance changes of living cells after irradiation [167, 170] clearly indicate that a mechanism based on changes in redox properties of terminal enzymes of respiratory chains might be crucial.

The primary physical and/or chemical changes induced by light in photo-acceptor molecules are followed by a cascade of biochemical reactions in the cell that do not need further light activation and occur in the dark (photo-signal transduction and amplification

chains). Usually, the irradiation lasts on a time scale of seconds and minutes. The biological responses of cells occurring later, when the radiation is switched off, are called secondary reactions.

These reactions are connected with changes in cellular homeostasis parameters. The crucial step here is thought to be an alteration of the cellular redox state: a shift towards oxidation is associated with stimulation of cellular vitality and a shift towards reduction is linked to inhibition. It was shown that cells with a lower than normal intracellular pH, in which the redox state was shifted in the reduced direction, were more sensitive to the stimulative action of light as compared to cells in which the respective parameters were optimal or near optimal. Any shift in the redox state of a cell will necessarily alter its radiosensitivity. It was observed that the pre-irradiation of cells with laser decreased their cytotoxic response to γ-radiation and it was taken as an additional proof of the theory that irradiation shifts the cellular redox state [154].

2.2.2 LLLT Applications

There are three main areas of medicine and veterinary practice where LLLT has a major role to play. These are (i) wound healing, tissue repair and prevention of tissue death; (ii) relief of inflammation in chronic diseases and injuries with its associated pain and edema; (iii) relief of neurogenic pain and some neurological problems [171].

Ozawa et al studied with rat calvarial cells *in vitro* to determine the target cells responsible for the action of laser irradiation and roles of irradiation on these cells during bone formation [180]. Ga-Al-As laser (830 nm, 500 mW) was used. Their results suggest that laser irradiation may play two principal roles in stimulating bone formation: stimulation of cellular proliferation, especially proliferation of nodule-forming cells of osteoblast lineage and stimulation of cellular differentiation, especially to committed precursors, resulting in an increase in the number of more differentiated osteoblastic cells and an increase in bone formation. Aihara et al applied Ga-Al-As to rat osteoclast precursor cells for 1, 3, 6, or 10 min at 24 h intervals during the culture period [176]. Their

results showed that LLLT facilitates differentiation and activation of osteoclasts via RANK (receptor activator of NF-kappaB) expression.

Stein et al investigated the effect of LLLT on proliferation and differentiation of human osteoblast cell line [178]. He-Ne laser (632 nm; 10 mW) was applied. A significant increase in cell survival and higher cell count in the once-irradiated as compared to non-irradiated cells was monitored. It was observed that enhancement of ALP activity and expression of OP and BSP was much higher in the irradiated cells as compared to non-irradiated osteoblasts. Hawkins et al also studied with He-Ne (632.8 nm) to establish cellular responses to laser irradiation using different laser fluences (0.5, 2.5, 5, 10, and 16 J/cm^2) with a single exposure on 2 consecutive days on normal and wounded human skin fibroblasts. Results showed that 5 J/cm^2 stimulates mitochondrial activity, which leads to normalization of cell function and stimulates cell proliferation and migration of wounded fibroblasts to accelerate wound closure. Study showed that laser irradiation can modify cellular processes in a dose (J/cm^2) dependent manner [184].

Forming cartilage tissue *in vitro* that resembles native tissue is one of the challenges of Cartilage Tissue Engineering. With this motivation, Gan et al studied to determine whether LLLT would improve the formation of cartilage tissue *in vitro*. Bovine articular chondrocytes were seeded on the top surface of porous calcium polyphosphate substrates. After 2 days, laser stimulation was applied daily at a wavelength of 650 nm using a laser diode with energy densities of either 1.75 or 3 J/cm^2 for 4 weeks [189]. Proteoglycan and collagen synthesis and matrix content were determined. Cartilage tissue morphology was evaluated histologically and observed that, there was no difference in the appearance or cellularity of the tissues that formed in the presence or absence of laser stimulation at either dosage. There were no differences in DNA content between treated and untreated constructs and live-dead assay confirmed that this treatment was not toxic to the cells. Laser stimulation at 3 J/cm^2 enhanced matrix synthesis resulting in significantly more tissue formation than laser stimulation at 1.75 J/cm^2 or untreated cultures.

In the orthopaedic field, the repair of articular cartilage is still a difficult problem, because of the physiological characters of cartilaginous tissues and chondrocytes. To find

an effective method of stimulating their regeneration, Jia et al focuses on a study on the biostimulation of rabbit articular chondrocytes *in vitro* by He-Ne laser [190]. The articular chondrocytes isolated from the cartilage of the femur of the rabbit were incubated and cultures were irradiated with laser at power output of 2-12 mW for 6.5 minutes, corresponding to the energy density of 1-6 J/cm^2. Laser treatment was performed three times at a 24-hour interval. After laser application, incubation was continued for 24 hours. Irradiation of 4-6 J/cm^2 increased the cell numbers and revealed a considerably higher cell proliferation activity comparing to control cultures. The energy density of 4 and 5 J/cm^2 remarkably increased cell growth with positive effect on synthesis and secretion of extracellular matrix. Their study showed that a particular laser irradiation stimulates articular chondrocytes. These findings also might be clinically relevant, indicating that LLLT is likely to achieve the repair of cartilage in clinic.

Lopes et al investigated whether LLLT can reduce muscular fatigue during tetanic contractions in rats. They were planned four groups receiving either one of three different LLLT doses (0.5, 1.0, and 2.5 J/cm^2) and a control group. Electrical stimulation was used to induce tetanic muscle contractions in the tibial anterior muscle. There was no significant difference between the 2.5 J/cm^2 laser-irradiated groups and the control group. Laser-irradiated groups (0.5 and 1.0 J/cm^2) had significantly longer contraction values than the control group. Laser groups receiving 0.5 and 1.0 J/cm^2 showed significant increases in mean performed work compared with both the control group and their first contraction values. Muscle damage was indirectly measured by creatine kinase levels in plasma. A distinct dose-response pattern was found in which 1.0 and 2.5 J/cm^2 LLLT groups had significantly lower creatine kinase levels than the 0.5 J/cm^2 LLLT group and the control group. They showed that doses of 0.5 and 1.0 J/cm^2 can prevent development of muscular fatigue in rats during repeated tetanic contractions.

Kreisler et al studied to evaluate a potential stimulatory effect of LLLT on the proliferation of human periodontal ligament fibroblasts (PDLF). Subconfluent monolayers were irradiated with an 809-nm diode laser (10 mW) operated at energy fluences of 1.96-7.84 J/cm^2. After laser treatment, the cultures were incubated for 24 h. The proliferation rate of the lased and control cultures was determined by means of fluorescence activity of a

reduction-oxidation (redox) indicator added to the cell culture. Proliferation was determined 24, 48 and 72 h after irradiation. The irradiated cells revealed a considerably higher proliferation activity than the controls. The differences were significant up to 72 h after irradiation. According to this study, a cellular effect of the soft laser application is clearly noticeable [203].

2.3 Low Level Laser Therapy for Stem Cells

Stem cells are currently in the news for two reasons: the successful cultivation of hESCs lines and reports that ASCs can differentiate into developmentally unrelated cell types, such as nerve cells into blood cells. The spotlight on stem cells has revealed gaps in the knowledge that must be filled to take advantage of their full potential for treating devastating degenerative diseases such as Parkinson's disease and muscular dystrophy. It is needed to know more about the intrinsic controls that keep stem cells as stem cells or direct them along particular differentiation pathways. Such intrinsic regulators are, in turn, sensitive to the influences of the microenvironment, or niche, where stem cells normally reside. Both intrinsic and extrinsic signals regulate stem cell fate and some of these signals have now been identified [207].

Vacek et al and Wang et al have studied the effect of LLLT on HSCs *in vitro*. These experiments showed there is indeed the effect of low intensity laser on the HSCs and the present effect is the promotion of HSCs proliferation. In other words, low intensity laser irradiation can act as an extrinsic signal regulating stem cell fate.

Vacek et al have studied the increase in the capacity of bone marrow exposed to He-Ne laser radiation for growth of GM-CFC colonies *in vitro* [204, 205]. They found single exposure of murine bone marrow cell suspension to He-Ne laser radiation elevates the potential of HSCs for growth of GM-CFC colonies *in vitro*, and the stimulatory action of He-Ne laser persists for 60 min after short-term irradiation.

Wang et al have studied the proliferative effect of low energy laser (510.6 nm, 5 J/cm^2) on human cord blood HSCs *in vitro* and the relationship between the effect of LLLT and colony stimulating factor (CSF) [206]. They observed that laser irradiation synergized with CSF in expansion of cord blood hematopoietic cell, the GM-CFUc number from the group treated with irradiation plus CSF increased significantly as compared with that from other groups including laser irradiation or CSF treatment alone or control. Reincubation of cord blood cells in the soft agar medium revealed that secondary colonies were formed and the absolute number of colony was obviously increased as compared with that before liquid culture, and the number of colony in irradiation group was more than these of non-irradiation group. Their experimental results showed that LLLT for cord blood stem cells is effective *in vitro* expansion of cord blood stem cells.

Another group suggested that the long-term effects of LLLT can involve mechanisms connected with activation of migration of stem cells towards damaged areas [199]. Stromal cell-derived factor-1 alpha (SDF-1) plays a critical role in stem cell migration towards areas of tissue injury and hypoxia. Gasparyan et al examined the influence of laser light on migration of stem cells (FDCP-mix stem cell line) in absence and in presence of SDF-1, using Transwell system with 2 connected chambers separated by a filter. The first chamber contained medium and stem cells, second chamber contained medium with or without SDF. Group 1 cells served as a control. Cells of groups 2 and 5 were irradiated by red diode laser (660 nm, 20 mW and 15 min). Cells of groups 3 and 6 received infrared (IR) diode laser (958 nm, 36 mW, and 15 min). Group 4 was SDF-1 control. Stem cells were seeded into first chamber of the Transwell system, and filters were placed into the wells containing medium with 150 ng/ml SDF-1 (groups 4-6) or without SDF-1 (groups 1-3). The count of spontaneously migrated cells was 100% in case of control. Red and IR laser light increased motility of stem cells up to 126% and 151% accordingly. Influence of SDF-1 caused directed migration of cells was 225%. Combined effects of light irradiation and SDF-1 were significantly stronger ($p<0.05$): 388% for SDF-1 and red laser, 427% for SDF-1 and IR laser. Study showed that laser radiation can up-regulate stem cell migration towards higher SDF-1 gradient.

Gottlib et al studied LLLT (632,8 nm He-Ne laser) with MSCs seeded on three-dimensional biomatrices [198]. The consequent phenotype modulation and development of

MSCs towards ossified tissue was studied in this combined biomatrix/LLLT system and in a control group, which was similarly grown, but was not treated by LLLT. The irradiated and non irradiated MSC were tested at 1–7, 10, 14, 21, 28 days of culturing via analysis of cellular distribution on matrices (trypan blue), calcium incorporation to newly formed tissue (alizarin red), bone nodule formation (von Kossa), fat aggregates formation (oil red O), ALP activity, scanning electron microscopy (SEM) and electron dispersive spectrometry (EDS). The results obtained from the irradiated samples showed enhanced tissue formation, appearance of phosphorous peaks and calcium and phosphate incorporation to newly formed tissue. Moreover, in irradiated samples ALP activity was significantly enhanced in early stages and notably reduced in late stages of culturing. These findings of cell and tissue parameters up to 28 days of culture revealed higher ossification levels in irradiated samples compared with the control group. This study suggested that both the surface properties of crystalline biomatrices and the LLLT have biostimulatory effects on the conversion of MSCs into bone-forming cells and on the induction of *ex-vivo* ossification.

To investigate the effects of the LLLT on stem cells *in vivo*, planarians under regenerative process were used by de Souza et al [197]. Amputated worms were divided into three study groups: a control group and two other groups submitted to daily 1 and 3 min long laser treatment sections at a power density of 910 W/m^2. A 685 nm diode laser (35 mW) was used. Samples were sent to histological analysis at the 4th, the 7th and the 15th days after amputation. A remarkable increase in stem cells counts for the fourth day of regeneration was observed when the regenerating worms was stimulated by the laser radiation. These findings also encourage further research works on the influence of LLLT onto stem cells and tissue regeneration.

At a very recent study published on April 2007 by Tuby et al, the effect of LLLT on the proliferation of BMSCs and cardiac stem cells (CSCs) was investigated (804 nm, Ga-As diode laser) [200]. Isolated MSCs and CSCs were cultured and laser irradiation was applied at energy densities of 1 and 3 J/cm^2. The number of MSCs and CSCs up to 2 and 4 weeks post-LLLT demonstrated a significant increase in the laser-treated cultures as compared to the control. This study clearly demonstrates the ability of LLLT to promote

proliferation of MSCs and CSCs *in vitro* and these results may have an important impact on Regenerative Medicine.

The above experiments shows that low intensity laser have a variety of biostimulatory effects and also can promote stem cell proliferation under the corresponding conditions. However there has been no sufficient work for optimum parameters of LLLT for different stem cell types or that studying laser biomodulation on stem cell differentiation yet. Moreover, as far as it is concerned, there has been no work on LLLT for ADMSCs.

3. OBJECTIVE

The goal of this study is to show the effects of LLLT on stem cells *in vitro*. The development and application of human stem cells offers a wide number of potential applications, including the prevention, diagnosis and treatment of human diseases. This is the most important motivation to use stem cells in the current research. Specifically, ADMSCs are considered to be the best to work with among MSCs; since they were first reported in 2001, their pluripotency, proliferative efficiency and low donor morbidity have been exceedingly confirmed.

A broad literature exists on LLLT with positive results for stimulation of various cell types. It is hypothesized that LLLT might also have a biostimulatory effect for the growth of ADMSCs *in vitro*. However there have been a few studies for LLLT on stem cells and although positive results of these studies, there have been no sufficient work for optimum parameters of LLLT for different stem cell types yet and need further investigations. Moreover, there has been no work on LLLT for ADMSCs.

This preliminary study was planned through a broad literature review that is described in detail in Chapter 2.

The wavelength and energy dose are two of the most important parameters of laser applications. Two different wavelengths (650 nm and 635 nm) were used here to assess wavelength dependent effects of LLLT for stem cells. 650 nm and 635 nm lasers are two of the widely used laser types for LLLT applications and since they are diode lasers, they are easier to study with cell cultures than He-Ne laser (632 nm) which is another widely used one for LLLT. Also, two different energy dose (1,6 J/cm^2 and 3,2 J/cm^2) were applied for both wavelengths in order to observe dose dependent effects and find more effective dose. These two doses are chosen according to the results of similar studies.

Most of LLLT studies are reported that the stimulatory effects are achieved by repeated irradiation on consecutive days rather than one application that using the same LLLT parameters. Thus, in this study irradiation was carried out on three consecutive days that was started 24 h (attachment of the cells on the plate surface) after seeding. Importantly, this procedure enables cells to stay less time outside of the incubator. It is also helpful to prevent temperature rise caused by the increase of laser energy on culture wells during irradiation procedure.

Cell thawing and cell culture procedures performed in this experiment, that are described in Chapter 4, are the most widely used procedures all over many laboratories and any specific changes were not done for LLLT application.

In the current study, the effect of LLLT on stem cells is investigated in terms of the enhancement in cell proliferation relative to control. Counting was done on four different time intervals (5^{th}, 9^{th}, 14^{th} and 21^{st} days) to be able to compare the effects of LLLT at various stages both early and late stages.

Results were statistically analyzed by both *t*-test (comparing only two groups between each other) and ANOVA (to see the group effect comparing both three groups: Laser-1, Laser-2 and control).

4. METHODS

4.1 Consent, Cell Derivation and Identification

The study was approved by İstanbul Memorial Hospital Internal Review Board. Adipose tissues were collected from volunteer patients with informed consent of them. After collection of tissue pieces, they were extensively washed with PBS and processed for enzymatic digestion using Collagenase Type-II. Final cell suspensions were seeded on tissue culture plates containing DMEM with 10 % FBS, 1 % penicillin-streptomycin and 2mM L-Glutamine. Cellular morphology was assessed by phase contrast microscopy. Immunological characterization was performed with flow cytometry against antibodies CD105, CD29, CD90, CD45, CD34, and CD44 [177]. Identified ADMSCs were cryopreserved on the second passage and showed similar growth rate upon thawing. It was observed that cryopreservation did not affect the multilineage potentials of MSCs. ADMCs identified as mesenchymal cells were cryopreserved and stored at liquid nitrogen for latter researches.

4.2 Cell Thawing and Cultivation

The cells were not thawed until proper media and plasticware were on hand. The vials of cryopreserved cells were removed from liquid nitrogen and incubated in a 37°C water bath. Cells cryopreserved at second passage were used for this experiment. They were closely monitored until the cells were completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells. During thawing, the cells were not vortexed in order not to give harm to cells. As soon as the cells were completely thawed, the outside of the vials were disinfected with 70% ethanol. In a laminar flow hood, 1 or 2 ml pipettes were used to transfer the cells to sterile 15 ml conical tubes. It is important not introduce any bubbles during the transfer process. Using a 10 ml pipette, 9 ml of medium (pre-warmed to 37°C) was slowly added to the 15 ml conical tube.

DMEM with 10% FBS, 1% 2 mM L- Glutamine and 1% Penicillin-Streptomycin was used as cell culture medium. The whole volume of medium was not added at once to the cells to prevent decreased cell viability due to osmotic shock. The cells suspension was mixed gently by slow pipetting up and down twice. It is important not to introduce any bubbles. The cells were not vortexed.

Table 4.1
Chemical and Biological Products used

Product	Company	Properties
Trypsin/EDTA Solution	Sigma	%0,05/%0,02 (w/v) in PBS w/o Ca^{+2}, Mg^{+2} <–20°*C* Volume:100ml
Penicillin-Streptomycin Solution	Sigma	10.000U/10.000 µg/ml +2 - +8 °*C* Volume: 50 ml
L-Glutamine	Sigma	200mM < -20°*C* Volume: 100 ml
Fetal Bovine Serum	Sigma	Mycoplasma tested Endotoxin tested < -20°*C* Volume: 100 ml
Minimum Essential Medium Eagle – Dulbecco Modification	Gibco	SterileA Includes 4.5 g/L Glucose No L-Glutamine, No Pyruvate Volume:500 ml
Phosphate Buffered Saline	AppliChem	Volume:250 ml

The tubes were centrifuged at 1000 rpm for 5 min to pellet the cells in order to remove residual cryopreservative (DMSO). As much of the supernatant as possible was decanted. The cells were resuspended into a suitable volume of medium (pre-warmed to 37°C). For T-25 tissue culture flask, 4 - 5 ml volume was used. The cells were incubated at 37°C in a 5% CO_2 humidified incubator.

Figure 4.1 Incubation of stem cells

The next day and every three to four days thereafter during cultivation medium (prewarmed to 37°C) was changed to fresh. Cell density was examined every day by inverted microscopy. When the cells were 90% confluent (7-10 days after seeding), they were sub-passaged. Second passage cells after thawing were used for this experiment.

Figure 4.2 Photographs of ADMSCs grown in T-25 culture flasks used in this study. (The left one shows cells a few days after seeding and the right one shows confluent cells a week after seeding.)

The day before laser irradiation, confluent cells at T-25 tissue culture flasks were rinsed twice with PBS and detached with 1 ml Trypsin - EDTA solution (0.25% Trypsin, 0.02% EDTA) per flask and incubated for 3 - 4 min in incubator. After detaching (cells were observed as rounded up under microscopy), medium with the same amount of Trypsin - EDTA solution (1 ml per flask) was added to flasks in order to stop enzyme activity and protect cells. The cells suspensions were added to the 15 ml conical tubes and were centrifuged at 1000 rpm for 5 minutes to remove enzyme solution.

After centrifugation, medium was added and cell suspensions were homogenized. Cells were counted and plated at a density 2 – 5 x 10^4 cells/ml in 96-well plates (Nunc). Three groups were constituted as control (C) and two laser treated groups with different laser fluencies as 3.2 J/cm^2 (L-1) and 1.6 J/cm^2 (L-2) to assess the effects of LLLT on these cells. The experiment is done with 650 nm diode laser firstly and repeated for the same fluencies with 635 nm diode laser. Two of the most important laser parameters for successful treatment are the energy density (fluence/dose) and wavelength.

4.3 Laser Irradiation

Irradiation was carried out using 5 mW diode lasers (Type: DI650-5-3 and Type: DI635-5-3, Huanic, China) (650 nm and 635 nm) with no distance between plate surface and probe (diameter of module probes above cell suspension and diameter of wells are both equal to 0.6 cm) (Figure 4.4). The irradiation durations of the irradiation groups were calculated using the equation below:

Fluence (J/cm^2) = Output power (mW) / Application Surface (cm^2) x Irradiation Duration (s)

The total irradiation durations for energy densities of 3.2 J/cm^2 (L1) and 1.6 J/cm^2 (L2) were calculated to be as 3 min (L1) and 90 s (L2). The energy densities used in this study, were selected at random below the therapeutic doses used in different cell cultures at previous studies.

24 h after plating in 96-well plates, cells were irradiated in the dark on days 1 (24 h after seeding), 2 and 3 (24 h between irradiations). 8 wells of every 96-well plates were used only, in order to avoid light dispersion to other wells.

Figure 4.3 8 wells used of a 96 well plate.

Irradiation durations per day were 60 s (L1 groups) and 30 s (L2 groups). After irradiation, cells were incubated (37°C) for tests. Cells were counted at Day 5 (2 days after post-irradiation), Day 9 (6 days after post-irradiation), Day 14 (11 days after post-irradiation) and Day 21 (24 days after post irradiation). All applications with cell cultures were performed under a laminar flow hood. During laser application, the lids of the plates were removed and the module probe placed above wells (equal diameters).

Power supply unit was not paced on the hood to distance it from the treatment specimen in order to limit any potential electromagnetic effects of the unit on the sample and ensure that effects are due to light delivery alone (Figure 4.4).

Figure 4.4 Laser Application.

The control plates were maintained under a laminar flow hood for a period of time corresponding to those of experimental groups but were not irradiated. Control and laser-irradiated plates were chosen at random.

4.4 Experimental Groups

There were 6 independent groups as 650-C, 650-L1, 650-L2, 635-C, 635-L1 and 635-L2 (Table 3.1). For every group there are 4 plates for counting at days 5, 9, 14, 21. Total plate number used was 24.

Table 4.2
Experimental Groups.

Group	**Control**	**Laser – 1 (L1)**	**Laser – 2 (L2)**	***Counting Days***
650 nm	No irradiation, for Day 5 Counting	Irradiation parameters: 650 nm, 1,6 J / cm^2 for Day 5 Counting	Irradiation parameters: 650 nm, 3,2 J / cm^2 for Day 5 Counting	5
	No irradiation, for Day 9 Counting	Irradiation parameters: 650 nm, 1,6 J / cm^2 for Day 9 Counting	Irradiation parameters: 650 nm, 3,2 J / cm^2 for Day 9 Counting	9
	No irradiation, for Day 14 Counting	Irradiation parameters: 650 nm, 1,6 J / cm^2 for Day 14 Counting	Irradiation parameters: 650 nm, 3,2 J / cm^2 for Day 14 Counting	14
	No irradiation, for Day 21 Counting	Irradiation parameters: 650 nm, 1,6 J / cm^2 for Day 21 Counting	Irradiation parameters: 650 nm, 3,2 J / cm^2 for Day 21 Counting	21
635 nm	No irradiation, for Day 5 Counting	Irradiation parameters: 635 nm, 1,6 J / cm^2 for Day 5 Counting	Irradiation parameters: 635 nm, 3,2 J / cm^2 for Day 5 Counting	5
	No irradiation, for Day 9 Counting	Irradiation parameters: 635 nm, 1,6 J / cm^2 for Day 9 Counting	Irradiation parameters: 635 nm, 3,2 J / cm^2 for Day 9 Counting	9
	No irradiation, for Day 14 Counting	Irradiation parameters: 635 nm, 1,6 J / cm^2 for Day 14 Counting	Irradiation parameters: 635 nm, 3,2 J / cm^2 for Day 14 Counting	14
	No irradiation, for Day 21 Counting	Irradiation parameters: 635 nm, 1,6 J / cm^2 for Day 21 Counting	Irradiation parameters: 635 nm, 3,2 J / cm^2 for Day 21 Counting	21

4.5 Cell Counting

For cell counting, the culture medium was removed from the wells and gently washed twice with PBS. The culture was separated afterwards using trypsin-EDTA solution (100 µl / well) for about 5 min. The cell suspension obtained was then added culture medium (100 µl / well) with the same amount of trypsin-EDTA solution. This suspension was used for statistical counts in a Neubauer chamber using an inverted microscope (Figure 3.5), randomly choosing four different areas and cell proliferation was expressed as number of cells / ml. All counting were repeated twice and average values were recorded.

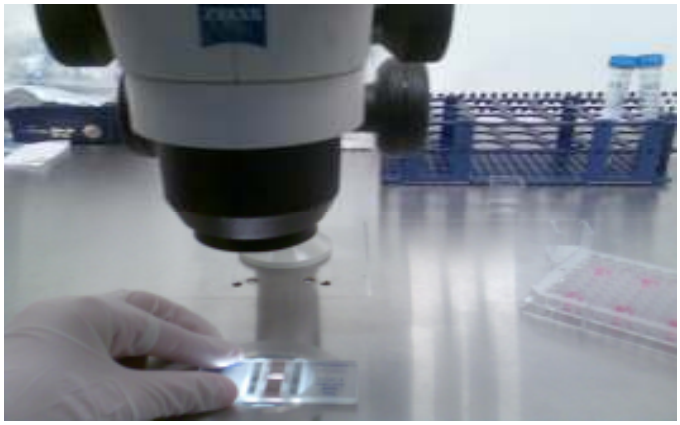

Figure 4.5 Cell counting.

4.6 Statistical Analysis

Mean values and standard deviations were calculated. An analysis of variance (ANOVA – One way) was carried out in order to assess the group effect, a possible effect of time, and an interaction between the 3 groups (C; L1; L2) at 4 time points (Day 5, 9, 14, 21) for laser applications with two different wavelengths. Values are expressed as means ± standard deviations (SD). P-values smaller than 0, 05 were considered statistically significant ($p < 0, 05$; $n = 8$). The mean values and SD values of the groups were used to plot their bar graphics.

Possible significant differences were assessed by means of the *t*-test. For *t*-test applications, a one-tailed test should be used if the hypothesis is that the mean of sample 1 is either higher or lower than the mean of sample 2; a two-tailed test should be used if the hypothesis is that the means of the two samples differ, no matter which one is higher and which is lower. Therefore, since it is hypothesized that laser groups should be higher than control groups, one-tailed test is used to compare laser groups with control groups. On the other hand, since it is not known that which dose is more effective, two-tailed test is used to compare laser groups with different doses between each other.

5. RESULTS AND DISCUSSION

Table 5.1 shows the data obtained from the Day 5 counting results of 650 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

Table 5.1

Counting Results (cells/ml) and Statistical Analysis for Day 5 of 650 nm laser.

Well	**C-650 nm**	**L1-650 nm (3,2 J/cm^2)**	**L2-650 nm (1,6 J/cm^2)**
1	50000	25000	47500
2	47500	17500	30000
3	42500	22500	40000
4	50000	17500	55000
5	42500	27500	37500
6	25000	35000	40000
7	30000	40000	47500
8	32500	20000	45000
Mean	40000	25750	42750
SD	9636,241	8210,403	7610,789

Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,0217	0, 263	0, 0028
St. Sign.	*Yes*	*No*	*Yes*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	9,349	3,467	*Yes*

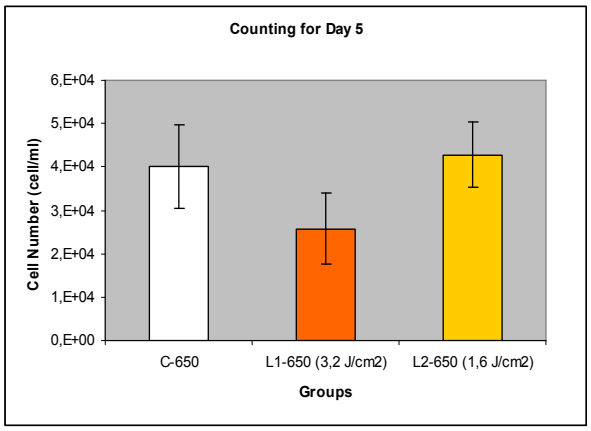

Figure 5.1 Counting results of 650 nm laser application for Day 5.

On Day 5 of 650 nm application, according to the *t*-test, there is a statistically significant difference between C vs L1 and L1 vs L2; L1-650 group is extremely lower than two groups. However there is not a statistically significant difference between C and L2 groups. ANOVA – One way test result gives that there is a statistically significant difference between three groups and they can be arranged as: L2 > C > L1.

Table 5.2 shows the data obtained from the Day 9 counting results of 650 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

On Day 9 of 650 nm application, according to the *t*-test, there is not a statistically significant difference between C vs L1, C vs L2 or L1 vs L2. ANOVA – One way test result gives that there is not a statistically significant difference between three groups.

Table 5.2

Counting Results (cells/ml) and Statistical Analysis for Day 9 of 650 nm laser.

Well	C-650 nm	L1-650 nm (3,2 J/cm^2)	L2-650 nm (1,6 J/cm^2)
1	57500	35000	52500
2	52500	35000	45000
3	47500	65000	40000
4	35000	35000	47500
5	45000	50000	37500
6	35000	40000	37500
7	42500	35000	37500
8	50000	60000	35000
Mean	45750	48250	42500
SD	7989,949	12374,37	6114,605

Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,402	0, 103	0, 645
St. Sign.	*No*	*No*	*No*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	0,409	3,467	*No*

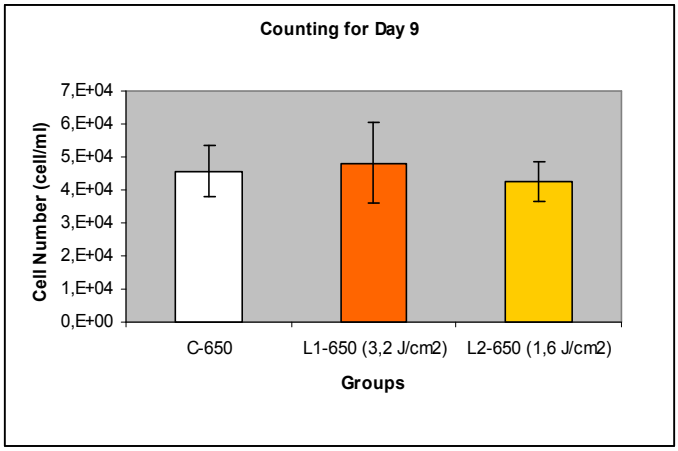

Figure 5.2 Counting results of 650 nm laser application for Day 9.

Table 5.3 shows the data obtained from the Day 14 counting results of 650 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

Table 5.3

Counting Results (cells/ml) and Statistical Analysis for Day 14 of 650 nm laser.

Well	C-650 nm	L1-650 nm (3,2 J/cm^2)	L2-650 nm (1,6 J/cm^2)
1	97500	97500	90000
2	95000	97500	107500
3	82500	112500	107500
4	70000	90000	90000
5	75000	72500	85000
6	70000	90000	87500
7	62500	82500	85000
8	60000	90000	95000
Mean	76562.5	91562.5	93437.5
SD	13296,08	12642,05	9970,194

Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,007	0, 003	0, 476
St. Sign.	*Yes*	*Yes*	*No*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	4,896	3,467	*Yes*

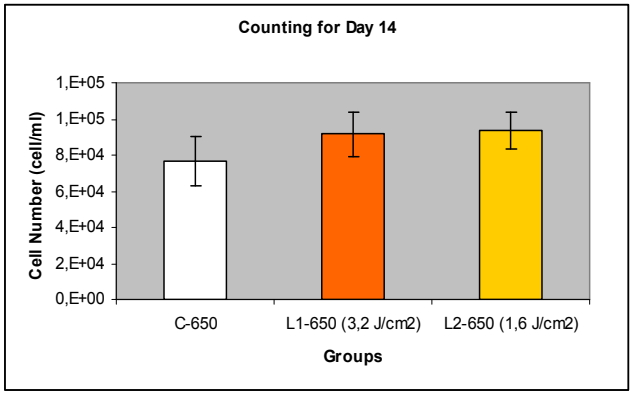

Figure 5.3 Counting results of 650 nm laser application for Day 14.

On Day 14 of 650 nm application, according to the *t*-test, there is a statistically significant difference between C vs L2 and C vs L1. There is not a statistically significant difference between L1 vs L2. ANOVA – One way test result gives that there is a statistically significant difference between three groups and they can be arranged as: L2 > L1 > C.

Table 5.4

Counting Results (cells/ml) and Statistical Analysis for Day 21 of 650 nm laser.

Well	**C-650 nm**	**L1-650 nm (3,2 J/cm^2)**	**L2-650 nm (1,6 J/cm^2)**
1	125000	97500	100000
2	112500	90000	97500
3	97500	112500	175000
4	132500	105000	132500
5	112500	90000	145000
6	137500	95000	95000
7	125000	102500	105000
8	97500	107500	120000
Mean	117500	100000	121250
SD	15059,41	8237,545	28157,72

Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,02	0, 396	0, 046
St. Sign.	*Yes*	*No*	*Yes*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	2,839	3,467	*No*

Table 5.4 shows the data obtained from the Day 21 counting results of 650 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

On Day 21 of 650 nm application, according to the *t*-test, there is a statistically significant difference between L1 vs L2 and C vs L1 but no a statistically significant difference between C1 vs L2. L1-650 group is extremely lower than two

groups similarly to results of Day 5. However, differently from Day 5, ANOVA – One way test result gives that there is not a statistically significant difference between three groups for Day 21.

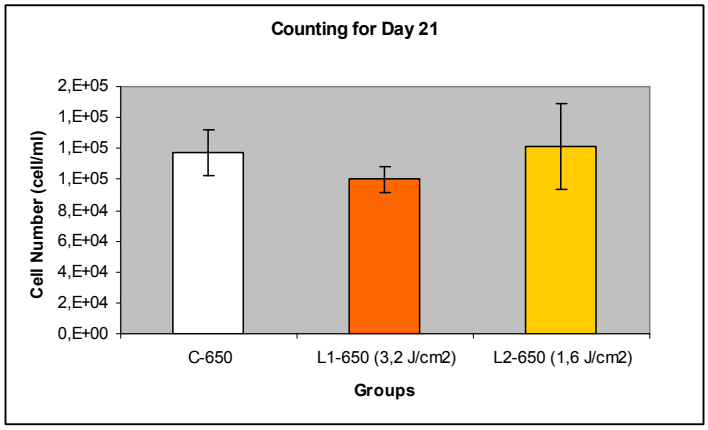

Figure 5.4 Counting results of 650 nm laser application for Day 21.

According to the overall results of 650 nm application, extent of enhancement of the cell proliferation by 650 nm diode laser irradiation was higher at 14 days post culturing than during the days of first week and third week. At Day 14, both two laser groups are significantly higher than control and L2 (1,6 J/cm^2) group is higher than L1 group (3,2 J/cm^2).

Table 5.5 shows the data obtained from the Day 5 counting results of 635 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

Table 5.5

Counting Results (cells/ml) and Statistical Analysis for Day 5 of 635 nm laser.

Well	C-635	L1-635 nm (3,2 J/cm^2)	L2-635 nm (1,6 J/cm^2)
1	65000	95000	82500
2	62500	92500	95000
3	55000	67500	92500
4	62500	75000	97500
5	47500	72500	95000
6	35000	67500	60000
7	45000	92500	50000
8	60000	70000	67500
Mean	54062,5	79062,5	80000
SD	10601,34	12096,15	18419,71

Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,0004	0, 0009	0, 91
St. Sign.	*Yes* **	*Yes* **	*No*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	8,687	3,467	*Yes*

On Day 5 of 635 nm application, according to the *t*-test, there is a statistically significant difference between C1 vs L2 and C vs L1. (**) P-values are not only smaller than 0,05 but also smaller than the value 0,001 which proves more significant difference than all other groups. However, there is not a statistically significant difference between L1 vs L2 according to the *t*-test. ANOVA – One way test result gives that there is a statistically significant difference between three groups for Day 5 and they can be arranged as: L2 > L1 > C.

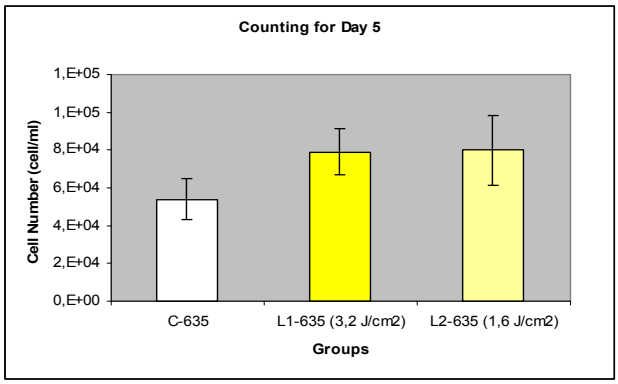

Figure 5.5 Counting results of 635 nm laser application for Day 5.

Table 5.6 shows the data obtained from the Day 9 counting results of 635 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

Table 5.6

Counting Results (cells/ml) and Statistical Analysis for Day 9 of 635 nm laser.

Well	**C-635 nm**	**L1-635 nm (3,2 J/cm^2)**	**L2-635 nm (1,6 J/cm^2)**
1	125000	105000	125000
2	120000	110000	115000
3	117500	100000	105000
4	100000	112500	132500
5	102500	125000	112500
6	110000	102500	102500
7	100000	122500	135000
8	102500	120000	100000
Mean	109687,5	112187,5	115937,5
SD	9949,65	9490,362	13557,91
Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,354	0, 182	0, 485
St. Sign.	*No*	*No*	*No*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	0,637	3,467	*No*

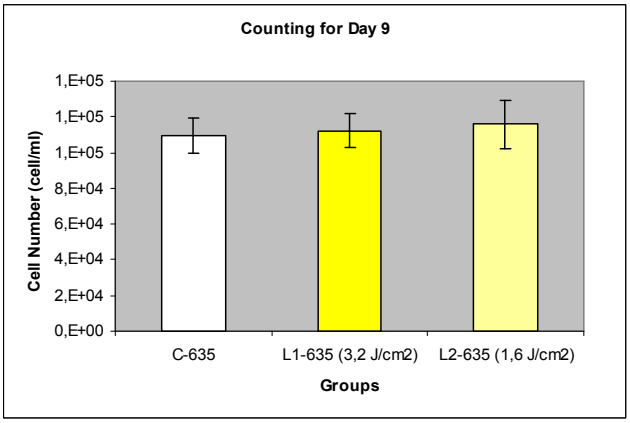

Figure 5.6 Counting results of 635 nm laser application for Day 9.

On Day 9 of 635 nm application, according to the *t*-test, there is not a statistically significant difference between C vs L1, C vs L2 or L1 vs L2. ANOVA – One way test result gives that there is not a statistically significant difference between three groups. It is important to specify that there were also no statistically significant difference On Day 9 results of 650 nm application.

Table 5.7 shows the data obtained from the Day 14 counting results of 635 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

Table 5.7

Counting Results (cells/ml) and Statistical Analysis for Day 14 of 635 nm laser.

Well	C-635 nm	L1-635 nm (3,2 J/cm^2)	L2-635 nm (1,6 J/cm^2)
1	155000	137500	172500
2	142500	150000	175000
3	175000	175000	132500
4	132500	132500	125000
5	150000	125000	170000
6	162500	112500	127500
7	135000	115000	125000
8	145000	110000	135000
Mean	149687,5	132187,5	145312,5
SD	14232,6	22056,64	22812,81

Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,019	0, 327	0, 22
St. Sign.	*Yes*	*No*	*No*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	1,646	3,467	*No*

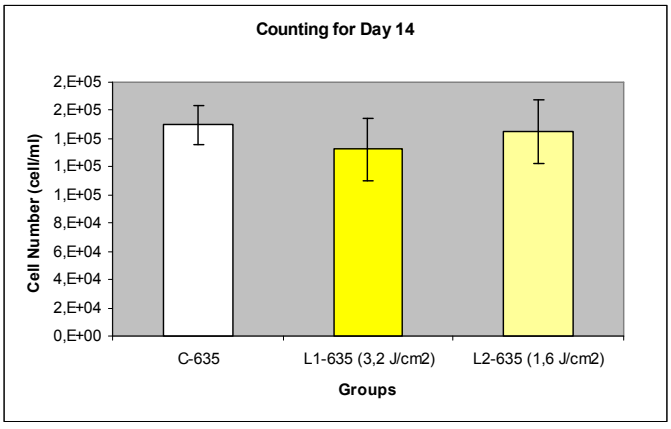

Figure 5.7 Counting results of 635 nm laser application for Day 14.

On Day 14 of 635 nm application, according to the *t*-test, there is a statistically significant difference between C vs L1 and L1-635 group is extremely lower than control

group. However there is not a statistically significant difference between C vs L2 and L1 vs L2 groups. ANOVA – One way test result gives that there is not a statistically significant difference between three groups for Day 14 counting of 635 nm application. It is important to note that L1-635 group showed an inhibited behavior here and similar effect was seen on Day 5 (and also on Day 21) counting result of L1-650 group which has the same energy dose (3,2 J/cm^2) with L1-635 group.

Table 5.8 shows the data obtained from the Day 21 counting results of 635 nm diode laser application with two different energy doses (3,2 J/cm^2 and 1,6 J/cm^2) and control. There are the counting results of every eight well of every group, group mean values, SD and statistical analysis of the groups.

Table 5.8

Counting Results (cells/ml) and Statistical Analysis for Day 21 of 635 nm laser.

Well	**C-635 nm**	**L1-635 nm (3,2 J/cm^2)**	**L2-635 nm (1,6 J/cm^2)**
1	182500	180000	160000
2	170000	187500	167500
3	157500	190000	172500
4	165000	155000	182500
5	187500	200000	162500
6	145000	157500	187500
7	172500	170000	187500
8	170000	180000	167500
Mean	168750	177500	173437,5
SD	13429,71	15754,82	11014,4
Statistical Analysis			
***t*-test groups**	**C vs L1**	**C vs L2**	**L1 vs L2**
P-values	0,0547	0, 287	0, 664
St. Sign.	*No* *	*No*	*No*
ANOVA	**F-value**	**Fcr-value**	**St. Sign.**
	0,837	3,467	*No*

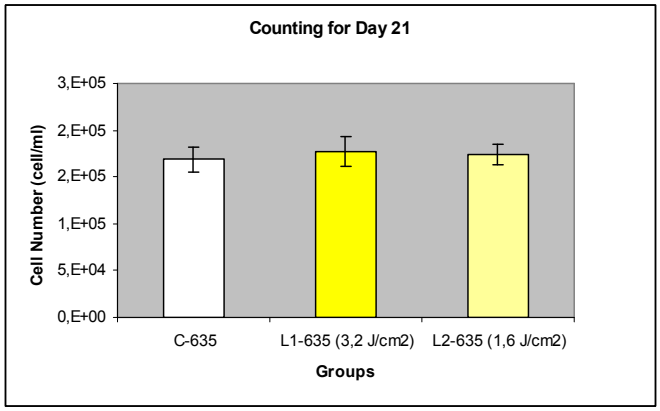

Figure 5.8 Counting results of 635 nm laser application for Day 21.

On Day 21 of 635 nm application, according to the *t*-test, there is not a statistically significant difference between C vs L1, C vs L2 or L1 vs L2 groups. (*) However P-value for C vs L1 (0,0547) is very close to the statistically significant value (0,05) which shows L1-635 is almost significantly higher than control group (C-635). ANOVA – One way test result gives that there is not a statistically significant difference between three groups for Day 21 counting.

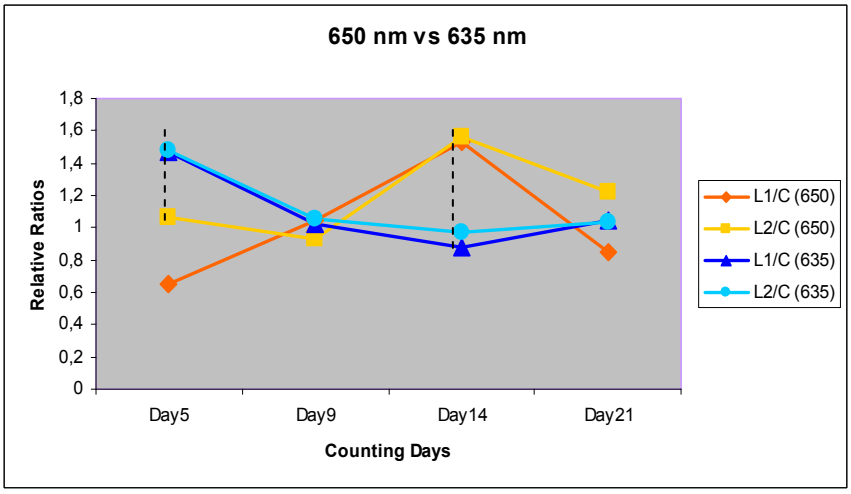

Figure 5.9 Proliferation rates of laser groups as relative to control on days 5, 9, 14 and 21.

Figure 5.9 shows the overall results of proliferation rates of laser groups with different wavelengths (635 nm and 650 nm) as relative to control on days 5, 9, 14 and 21. Peak values can be seen for 650 nm application on Day 5 and for 635 nm application on day 14. On days 9 and 21, groups are close to each other and control.

Table 5.9

Overall ANOVA results for all counting days.

Days	650 nm	635 nm
5	**L2 > C > L1 †**	**L2 > L1 > C**
9	L2 ≈ L1 ≈ C	L2 ≈ L1 ≈ C
14	**L2 > L1 > C**	L2 ≈ L1 ≈ C
21	L2 ≈ L1 ≈ C	L2 ≈ L1 ≈ C

Table 5.9 clearly demonstrates the counting day effect with different wavelengths. For 650 nm application there is a statistically significant difference on Day 5 and for 635 nm irradiation, it can be seen on Day 14. On all other days for both wavelengths there is not a statistically significant difference except Day 5 counting of 650 nm. (†) On Day 5, L1-650 group was extremely lower than other laser group (L2-650) and control group (C-650). Therefore, ANOVA result of the three groups calculated as statistically significant although the values of other two groups are not statistically significant between each other (*t*-test results, Table 5.1).

A number of studies have demonstrated the effect of LLLT on stem cells *in vitro* [204 -206, 200 - 198] and *in vivo* [197]; but the optimum radiation parameters for specific stem cell types are still unclear and there is no study on biostimulation of ADMSCs with LLLT. LLLT application can also be beneficial to enhance the proliferation of ADMSCs *in vitro*.

In the present study, ADMSCs culture was used to assess the effects of laser irradiation on stem cells *in vitro* and it is demonstrated for the first time that LLLT significantly promotes the growth of ADMSCs. There are significantly different results as compared to the control group for both laser groups with different wavelengths (650 nm and 635 nm) and fluencies (1,6 and 3,2 J/cm^2).

One of the most important results of this study is time dependent effects of different wavelengths. The extent of enhancement of the cell proliferation by 650 nm diode laser irradiation was significantly higher at 14 days post culturing than the enhancements during the first week and third week (Figure 6.1). These results confirm those of previous studies suggested that light may have a very long term effect and induce late expressing alteration in cell activity. For instance, Dörtbudak et al reported that the proliferation of osteoblasts derived mesenchymal cells irradiated with 690 nm laser was significantly higher than control only after 12 days post culturing but not before [26]. Similarly, Tuby et al showed that proliferation of BMSCs by laser irradiation (804 nm, Ga-As diode laser) was higher at 3 weeks post culturing than during the first two weeks [200]. Byrnes et al studied another property except proliferation and they observed that laser irradiation (810 nm) resulted in gene expression alterations radically on 21st day, with no significant change on 7th day [181].

On the other hand, the increase in the cell growth by 635 nm diode laser application relative to non-irradiated cultures was significantly higher on 5th day but not during the second week and third week (Figure 6.1). These results are similar to those of previous studies that demonstrated enhancement of cell growth of various cell types *in vitro* by laser irradiation within the first few days post laser application. Likewise, Moore et al observed an increase in proliferation of both fibroblast and endothelial cells with various wavelengths only on day 4 post culturing [183]. Gottlib et al showed that laser irradiation (632,8 nm He-Ne laser) enhanced the ALP activity and the conversation of BMSCs into osteoblastic phenotype in the early stages (Day 2) [198]. Khadra et al also studied with osteoblasts and reported that the number of irradiated (830 nm, GaAlAs diode laser) osteoblasts was significantly higher than control group after 7 days post culturing [179]. Jia et al focuses on a study on the biostimulation of rabbit articular chondrocytes *in vitro* and they showed that irradiation (He-Ne laser) revealed a considerably higher cell proliferation activity comparing to control cultures on day 5 [190]. Planarians under regenerative process were used by de Souza et al to show the effects of the LLLT (685 nm diode laser) on stem cells *in vivo* [197]. Samples were analyzed at the 4th, the 7th and the 15th days after irradiation. A remarkable increase in stem cells counts for the fourth day of regeneration was observed when the regenerating worms was stimulated by the laser radiation with no significant differences on days 7 and 15.

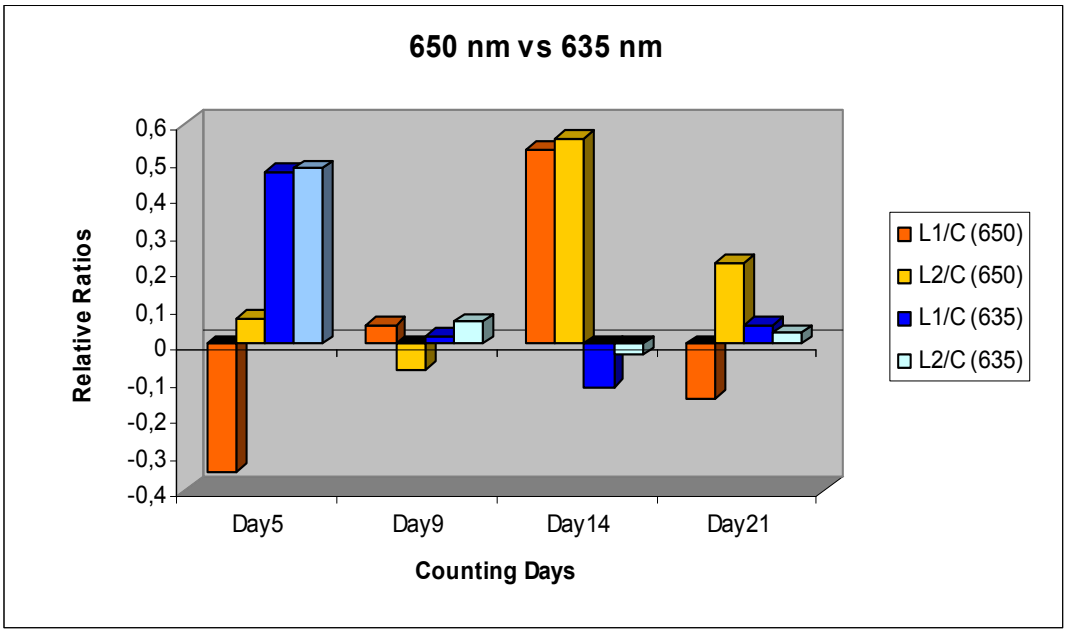

Figure 5.10 Proliferation rates of laser groups as relative to control on days 5, 9, 14 and 21.

The fact assessed in this study that irradiating the same type of cells with varied wavelengths results in different enhanced proliferation stages, may be useful. By this way, different laser protocols can be used for several applications. 650 nm might be used for the long term applications with ADMSCs, like *in vitro* studies going on laboratories. And 635 nm can be beneficial to enhance the proliferation of ADMSCs from a donor patient and used for implantation within a short time interval. In an injured organ, the time of implantation post-injury may be crucial to achieve maximum beneficial clinical effect. Moreover, since LLLT has been shown to induce a cytoprotective effect on cells (increase in heat shock proteins [174], antioxidants [174] and antiapoptotic activity [173]), the laser irradiated cells may have a better long term survival post-implantation and can serve better as stem cells for production of growth factors in the host organ or participate in the regenerative process.

Karu pointed out that only the proliferation of slowly growing subpopulations can be stimulated by irradiation and it is not possible to activate a process which is activated already or occurring at speed near maximal [187]. In this case, the process which is

activated already or occurring at speed near maximal is the process of the cellular state in homeostasis. The cells might be not in homeostasis on the 5^{th} or 14^{th} day just before confluency (it was observed that cells became confluent 7-10 days after seeding). And may be, that is why they are much more affected relative to control on these days only.

These results support the study of Liu et al which states that LLLT might modulate the disruption of biosystem homeostasis until it was restored or a new homeostasis was established [172]. Therefore, the stimulated proliferation of slowly growing subpopulations by irradiation might be higher than other stages (Figure 5.2). Furthermore, on Day 9, all laser groups show similar characteristics to control and there is no significant difference. These results may represent that the cells are in homeostasis on Day 9 (Figure 5.2).

It is unclear whether the same mitogenic stimulus is created by each wavelength or if there is difference in mechanism of cell stimulation. The current results, with time dependent wavelength effect, might be a sign for the claim of different mechanisms. Molecular biology studies are warranted to determine if there are differences in molecular responses by cells exposed to various wavelengths of laser light. These studies will help elucidate the mechanisms of laser biostimulation and may help to identify new mitogenic pathways allowing for the development of novel therapies.

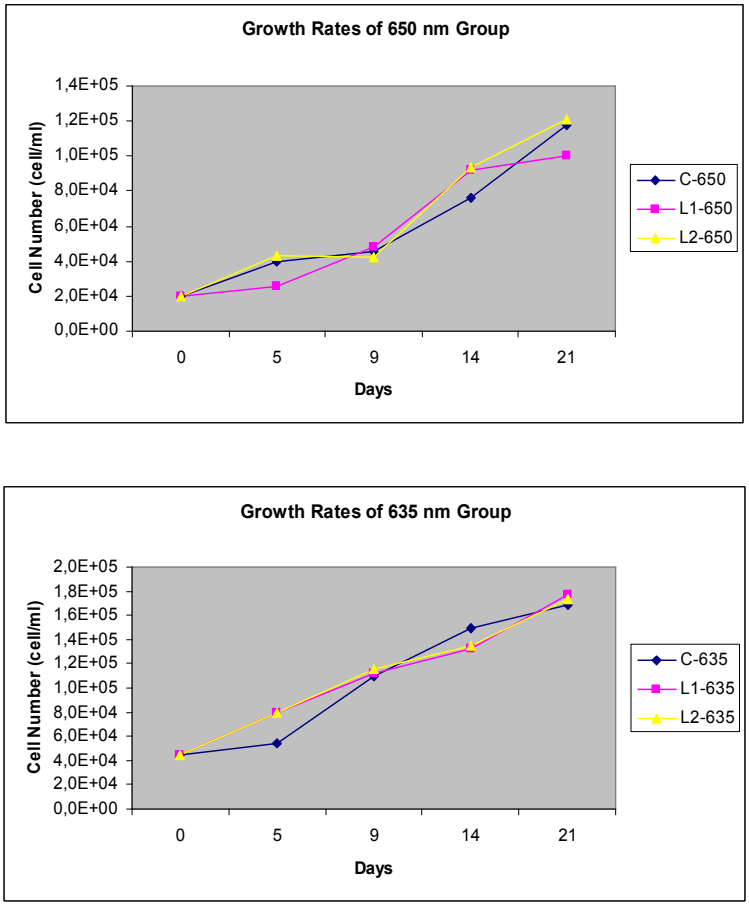

Figure 5.11 Growth curves of the experimental groups with wavelengths versus cultivation days.

Most of the previous studies report the proliferation rates for only one time interval (at mostly early or late stage). For example, Moore et al presented only cell counting results on Day 4 [183]; Jia et al [190]and Quero et al [182] on Day 5; Khadra et at on Days 2, 3 and 4 [179]. Here, these rates were observed for four different time intervals which ensure comparing the effects of LLLT at various stages that enhance the value of the study. The results of one study which appear like successful for only one time interval (early or late), may show negative effects on other days that are not tested.

In the current study, wavelength (650 nm and 635 nm) and energy density (1,6 J/cm^2 and 3,2 J/cm^2) are variables and two different values were chosen for each. Nevertheless; output power, exposure time, irradiation period and irradiated area were held constant. However, these are additional variables that may affect cellular response to laser

irradiation. For instance; it is demonstrated with previous studies that, little differences in energy density can change cell growth, which is consistent with theories stating that LLLT can stimulate cell proliferation but only within combination of parameters in a narrow energy and power density bands. Therefore, there may be more optimum parameters than used ones in this study that will show the LLLT effects on these cells.

From the current findings, it would appear that there is not a significant difference between two energy density groups (1,6 J/cm^2 and 3,2 J/cm^2) for the same wavelength groups (635 nm or 650 nm). On the other hand, the energy density of 1,6 J/cm^2 did not caused inhibition of cell proliferation oppose to 3,2 J/cm^2 in both wavelengths for ADMSCs. 1,6 J/cm^2 may be preferred for further studies. This finding is similar to some of the previous studies with different cell types. Dörtbudak et al used 1,6 J/cm^2 for bone marrow cells and found significant positive results [32]; Quero et al stated that 1,04 J/cm^2 was the most effective dose that they found for mammary adenoma cells [182]; Tuby et al showed that 1 J/cm^2 is more effective than the dose 3 J/cm^2 for BMSCs and CSCs [200]. Since, this is the first LLLT study with ADMSCs, it remains to be seen if energy doses other than that used in this study (1,6 and 3,2 J/cm^2) have a different effect on the proliferation of ADMSCs and what the most effective dose is. For the right dose, numerous parameters should be taken into account like wavelength and output power and much more experimental studies should be performed.

Most of LLLT studies are reported that the stimulatory effects are achieved by repeated irradiation on consecutive days rather than one application using the same LLLT parameters: Jia [190], Khadra [179] and Byrnes [181] et al applied irradiation for 3 consecutive days; Quero et al 4 consecutive days [182]; Ozawa et al applied for 15 consecutive days [180]; Dörtbudak et al irradiated cells on days 3, 5 and 7 [32] and Hawkins et al for 2 consecutive days [184]. Thus, irradiation was done on three consecutive days in this study. It is also helpful to prevent temperature rise caused by the increase of laser energy on culture wells during irradiation procedure.

Except one group for only one time interval, there was not a significant decrease relative to control which may be viewed as cell inhibition. The cells showed a good growth

behavior without any deficiency symptoms during the entire observation period (Figure 6.2). Counting results of Day 5 showed that laser group with the wavelength 650 nm and energy density 3,2 J/cm^2 (650-L1) is significantly less than control group and other laser groups; however following days the cell number increased. 650 nm diode laser with 3,2 J/cm^2 may cause cellular inhibition for ADMSCs at early stages of proliferation and not a permanent effect. Besides, this inhibition can be considered as experimental error.

MSCs are considered as very important in Cell Therapy, Bioengineering and Regenerative Medicine. Specifically, ADMSCs can be induced into multiple lineages in specific culture systems similar to bone marrow and umbilical cord MSCs [113]. Moreover, ADMSCs are much more abundant, easier to obtain, there is much less cell heterogeneity and also adipose tissue has a large number of cells. They are also capable of expressing multiple growth factors. Therefore, it is believed that ADMSCs represent an ideal substitute for other stem cells used in Cell-based Tissue Engineering. The results of the present study may therefore have clinical relevance. This is the first LLLT study done with ADMSCs, therefore parameters used and results also have scientific impact.

Since MSCs are pluripotent, further investigations should take place of the effect of LLLT on MSCs phenotypic distribution, behavior, differentiation and other biochemical pathways. Histochemical analysis, electron microscopy analysis and microarray analysis might be done to be able to investigate other effects of LLLT on the growth of MSCs and to achieve more clear results. The mechanisms associated with this phenomenon and the most effective irradiation parameters remain to be elucidated by further studies.

6. CONCLUSION

In the present study, ADMSCs culture was used to show the effects of laser irradiation on stem cells *in vitro*. It is demonstrated for the first time that LLLT significantly promotes the growth of ADMSCs. There are significantly different results as compared to the control group for both laser groups with different wavelengths (650 nm and 635 nm) and fluencies (1,6 and 3,2 J/cm^2).

The results of this study demonstrated that there is a time dependent effect of different wavelengths on ADMSCs. It was observed that the extent of enhancement of the cell proliferation by 650 nm diode laser was significantly higher relative to control group at 14th day. On the other hand, 635 nm diode laser application results showed significantly higher growing enhancement relative to control group on 5th day. These findings may be useful for different types of clinical applications with ADMSCs.

There was not a significant decrease relative to control which may be viewed as cell inhibition (except one group for only one time interval). The cells showed a good growth behavior without any deficiency symptoms during the entire observation period.

From the current findings, it would appear that there is not a significant difference between two energy density groups (1,6 J/cm^2 and 3,2 J/cm^2) for the same wavelength groups (635 nm or 650 nm). On the other hand, the energy density of 1,6 J/cm^2 did not caused inhibition of cell proliferation oppose to 3,2 J/cm^2 in both wavelengths for ADMSCs. 1,6 J/cm^2 may be preferred for further studies.

It is believed that ADMSCs represent an ideal substitute for other stem cells used in Cell-based Tissue Engineering. The results of the present study may therefore have clinical relevance. This is the first LLLT study done with ADMSCs; so parameters used and results also have scientific impact.

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