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Evaluation of critical parameters of low level laser irradiation on human osteoblast cell proliferation and differentiation

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STUDENT SIGNATURE:_____

Samuel Dean Waddoups, D.D.S. Date

EVALUATION OF CRITICAL PARAMETERS OF LOW LEVEL LASER IRRADIATION ON HUMAN OSTEOBLAST CELL PROLIFERATION AND DIFFERENTIATION

A Thesis Presented
By
SAMUEL DEAN WADDOUPS, D.D.S.

Submitted to the College of Dental Medicine of Nova Southeastern University in partial fulfillment of the requirements for the degree of

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Dedication

I would like to dedicate this thesis to my wife, Emily and my two children, who have inspired and supported me throughout this process. In addition, I would like to dedicate this thesis to my parents, who inspired and taught me sound principles that have blessed and guided me throughout my life. It has always been about family and it always will be!

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EVALUATION OF CRITICAL PARAMETERS OF LOW LEVEL LASER IRRADIATION ON HUMAN OSTEOBLAST CELL PROLIFERATION AND DIFFERENTIATION

DEGREE DATE: DECEMBER 7th, 2012

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SYNOPSIS

Orthodontic tooth movement is a biological response to a mechanical force. One of the challenges in orthodontics is obtaining desired tooth movement during treatment. Accelerating tooth movement and decreasing demands on anchorage can reduce treatment times and overall satisfaction for patient and doctor. Low-level laser therapy (LLLT) is emerging as a technology that may decrease orthodontic treatment time. Many *in vitro* and in *vivo* studies have reported the effects of low level lasers at random time points and energy densities. None of the studies have optimized the dose required for osteoblast proliferation and differentiation.

The purpose of this study was to find the optimum stimulatory dose of low level laser irradiation (LLLI) on human osteoblast cell proliferation and differentiation and to analyze our findings with reference to the Arndt-Shultz Law of applied energy. In this *in vitro* study a GaAlAs laser at 830nm, 20 mW with continuous exposure at various doses were used on a human osteoblast cell line. According to the Arndt-Shulz Law weak stimuli initiate vital activity, moderate stimuli enhance the cellular activity with subsequent peak stimulation and greater stimuli (beyond a threshold value) may not have any influence or inhibit the vital activity. The implications of LLLI on human osteoblasts and influencing tooth movement in orthodontics were discussed.

Human osteoblasts were cultured in minimum essential medium (MEM) complete medium consisting 10% fetal bovine serum and 1% antibiotics. Cells grown in complete medium were plated onto 96 well plate, allowed to adhere for 4-5 hours and were exposed to GaAlAs lasers at 6 , 12, 18, 24, 30, 36, 45, 60, 75, and 90 seconds. The cells treated with

LLLI were assessed for cell proliferation at 24, 48 and 72 hour intervals. A calorimetric cell proliferation assay (WST-1) assay was performed according to manufacture's instructions.

The results indicated that at 24 hours the 6 and 12 seconds doses significantly inhibited proliferation compared to the control. At 48 hours the 30 seconds exposure significantly increased proliferation. At 72 hours time interval, cell proliferation was observed in a dose dependent pattern with a minimum at 6 seconds with peak proliferation at 18 seconds. A gradual decrease in cell viability was observed in the cells treated beyond this dose with a maximum inhibition seen at 60 seconds. At 75 and 90 seconds no difference was observed between the control and experimental group.

To establish efficient acquisition of adequate quantities of alkaline phosphatase, cells were grown in 12 well plates in complete medium or osteogenic medium. These cells were exposed to LLLI for 18, 48, and 60 seconds. The activity of early osteogenic differentiation marker alkaline phosphatase (ALP) was investigated 10 days post exposure.

Our results demonstrated that alkaline phosphatase activity at 2.4 – 7.3 J/cm^2 with 48 – 60 seconds of exposure, and an incident power ranging from 85-269mw significantly increased. The findings suggest that these irradiated cells obeyed the Arndt Shulz Law governing cellular response to applied energy. Further this research indicates the possible role of LLLT to accelerate tooth movement in orthodontics. Complete disclosure of low level laser parameters is essential in order to accurately compare findings of researchers.

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Chapter 1: Introduction

1.1 Background

Recent advances in orthodontic procedures offer patient friendly treatment. In today's age of immediate gratification from the increased flow of information and convenience the population demands results faster and better than ever before in history. One of the challenges in modern orthodontics is to satisfy that demand. Unfortunately, the tooth can only move safely through the alveolus at the speed with which the surrounding biology allows. Orthodontic tooth movement is a biological response to a mechanical force. Many of the brightest minds in orthodontics have pondered upon how to manipulate forces by using innovative mechanics to create desired tooth movements, ultimately reducing treatment time. Some of these mechanisms include differential moments, functional and orthopedic appliances, temporary skeletal anchorage devices, elastics, magnets and many more. The manner in which the force is applied to the tooth to obtain the desired movement has been deeply studied and is well understood by the diligent orthodontist.

The biological manipulation of the biological-mechanical process in tooth movement is the new frontier for reducing treatment time. The biological part of the process has been less studied, is not as well understood, and offers the greatest opportunity for improvement. Emerging methods to effect the biological part of tooth movement, include but are not limited to, electric stimulation[1], ultrasound application[2] and drug injections[3] including prostaglandins[4], osteocalcin[2], relaxin[5], and the active form of vitamin D[6]. These methods appear to stimulate the rate of tooth movement but the drug injections may have side effects such as pain and discomfort, the other methods including electric stimulation and ultrasound are not commonly

used in an orthodontic clinic. Lasers, however, are beginning to be used more commonly in dental practice.

Low-Level Laser Therapy (LLLT) is beginning to emerge as a technology that may decrease orthodontic treatment time[7]. LLLT was recently highlighted on the cover of the March 2012 issue of the American Journal of Orthodontics and Dentofacial Orthopedics referring to an article finding that LLLT has the capacity to decrease pain and treatment time on human subjects[8]. Low level laser irradiation (LLLI) has also been found to stimulate the formation of osteoclasts on the pressure side during experimental tooth movement, increasing the rate tooth movement in rats[9]. It is believed that LLLI affects the proliferation of osteoblast cells that build bone but it is not yet clearly understood how the laser acts on the cells[10]. Increased activity of these cells responsible for bone remodeling may allow faster tooth movement while decreasing or inhibiting these cells could decrease tooth movement and improve anchorage. Controlling the rate of tooth movement through the alveolus during orthodontic treatment would enhance treatment time.

Very little is known about the optimum doses of laser irradiation on human osteoblasts because previous studies reported randomly energy densities, time points and power output. None of the studies reported the use of an optimum dose with reference to cellular activity. It is very important to establish an optimal dose as the cellular activity depends on the amount of stimulation.

Orthodontics is concerned with tooth movement in humans and so this research examines the effects of varying doses of LLLI to the human osteoblast using proliferation and ALP *in vitro* to find optimum inhibition and proliferation in vitro. The findings will be compared to the Arndt-

Shulz law governing cellular response to applied energy which suggests that if insufficient energy is applied to the cells no response will occur, with more energy a threshold will be crossed and biostimulation (cell proliferation and differentiation) should occur, with ever increasing energy the cells should reach a climax and then begin to be inhibited. In this study, I intend to evaluate the effects of LLLI on osteoblast proliferation and differentiation.

1.2 Biology of Tooth Movement

Current challenges in orthodontic therapy include the human body's capacity to remodel periodontal tissues and resorb and form new bone as tooth move through the alveolus. The tooth is suspended within the bone attached to periodontal ligaments (PDL). When forces are applied on the dentition these ligaments transfer the force from the tooth to the periodontal ligament and on to surrounding bone. When the force is administered for a significant duration of time the osteoclasts resorb bone on the pressure side of teeth providing a space into which the tooth to move. Osteoblasts build bone on the tension side of teeth as they move through the alveolus.[11, 12] Figure 1 depicts the concept of tooth movement through the alveolus.

Figure 1. Areas of Tension and Osteoblast Activity Coincide in Tooth Movement

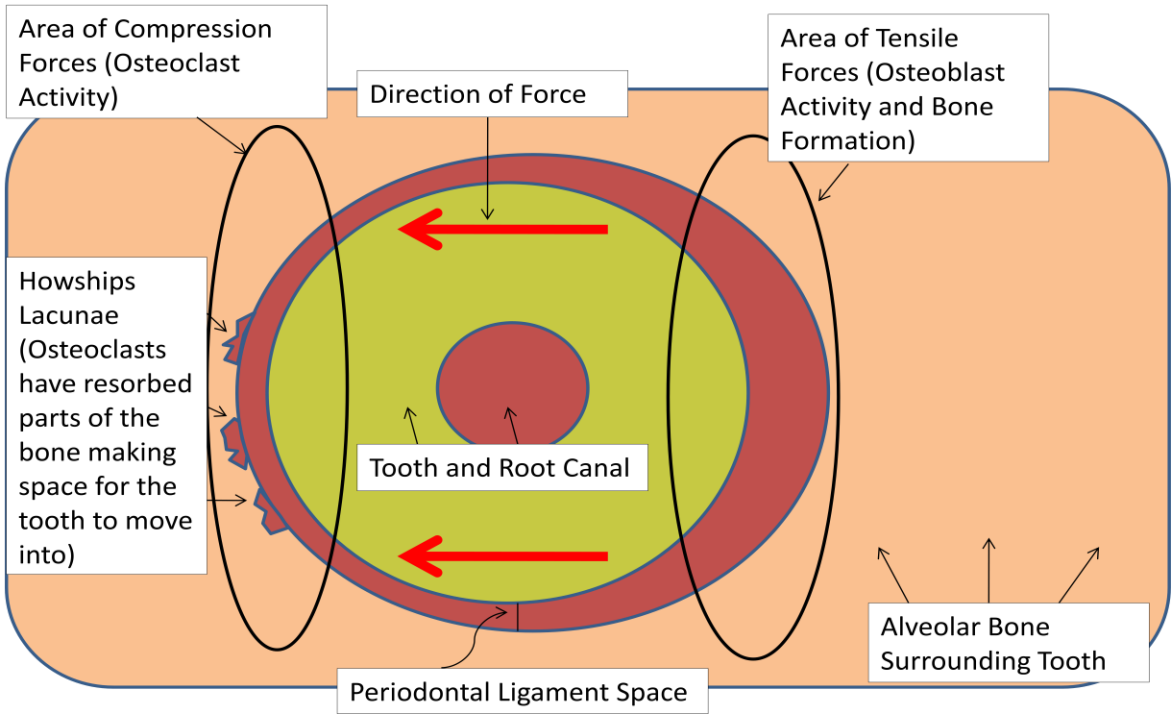


Figure 1 demonstrates how a mechanical force can affect a biological process, thus "biomechanics". The circled area on the right of the figure encompasses the area of high tensile forces and the area you would find high osteoblast activity[13]. The two red arrows indicate the direction of the force on the tooth. The tooth is being moved bodily to the left. Osteoclasts and Howships Lacunae (resorbed areas of bone) are seen on the pressure side (the circled area on the left) of the tooth creating space for the tooth to move in the direction of force[11]. Osteoblasts and new bone formation are seen on the tension side of the tooth being moved, filling in the void created by tooth movement. The tensile forces on the periodontal ligament space (PDL) right of the tooth root demonstrate an area of increased osteoblast activity and dilated blood vessels. Compression forces on the PDL are seen at left with increased osteoclast activity and compressed blood vessels.[12]

Osteoblasts and Osteoclasts

Osteoblasts are stromal cells that synthesize and deposit calcium phosphate crystals, hydroxyapatite, to form bone. Osteoblasts are differentiated from mesenchymal cells that differentiate into osteoprogenitors, then pre-osteoblasts and finally the mature osteoblast. These cells differentiate and proliferate by interaction with a large and complex group of cytokines and molecules. Pre-osteoblasts do not form bone matrix and have limited dividing capability. Once osteoblasts create the bone matrix a few will embed in that matrix and become osteocytes.[14] These osteocytes do not create or destroy bone but it is believed that they can be reactivated into bone production[15].

Osteoclasts are large, multinucleated, and terminally differentiated cells and are usually found on the endosteal, or periosteal, surface lining the bone. Osteoclasts come from the macrophage or mononcyte cell line. Osteoclasts degrade bone tissue creating areas of bone resorption.[14] Interestingly, there is evidence that osteoblasts are essential for osteoclasts to resorb bone[16]. Unlike the osteoblast the osteoclast does not progress further to an osteocyte but is terminally differentiated meaning that once the macrophage stops resorbing bone it undergoes apoptosis[17].

Osteoblasts regulate the development, differentiation, and function of the osteoclasts. Osteoblasts produce a protein called osteoprotegrin (OPG), also known as osteoclast inhibitory factor (OCIF) that is believed to inhibit osteoclastogenesis. The osteoblast also contains a transmembrane protein called osteoprotegrin ligand (OPGL) that is also known as a receptor activator of NFkB ligand (RANKL). [14] The osteoblast has many other pathways that it can communicate with the osteoclast. The intriguing part about the osteoblast is that it has the capacity to increase and decrease osteoclastic development, differentiation, and function.[14]

The above explanation of the osteoblast and osteoclast relationship demonstrates to the reader that the cell to scrutinize, in order to unlock the potential for increased tooth movement through the alveolus, is the osteoblast. If the LLLI can stimulate the osteoblast then it in turn has the power to influence the osteoclast. With increased control of these two cell lines much could be obtained for the advancement of not only orthodontics but the entire medical field involved with bone and bone pathology. In this thesis I intend to evaluate the effects of laser light on the osteoblast in particular low level laser light.

1.3 Lasers

Laser is an acronym for "Light Amplification by the Stimulated Emission of Radiation". Laser irradiation occurs when a photon is emitted prematurely from an over stimulated atom. A photon is the basic unit of light and has both electromagnetic wave, and particle characteristics. The photon then interacts with the other excited atoms by decaying their excited electrons to a lower energy state causing release of a second photon. This stimulated emission of the second photon (resulting in the start of a laser) can only occur if the incident photon has exactly the same energy as the released photon resulting in two identical photons in wavelength, direction, and phase. If there are more atoms in an excited state these 2 photons can then interact to create 4, 8, 16, 32 and so on, resulting in laser light. The wavelength is the physical property that determines the classification of electromagnetic energy. For example, gamma rays, x-rays, ultraviolet rays are considered to have very short wavelengths. Infrared, microwaves, and radio waves have longer wave lengths. [18] Figures 2 and 3 demonstrate the basic components of the conventional optical resonance laser and the diode laser. In this study a GaAlAs diode laser was used.

Laser light has multiple parameters that attempt to describe it, some of which are: wavelength, power density, pulse, coherence, and polarization. Wavelength is measured in nanometers (nm). Power density is measured in Watts/cm^2 or milliwatts (mw). Pulse duration is measured in units of time, but in this study continuous laser irradiation was used. Coherence and polarization are laser light parameters that are inherent. The energy, or dose, that is delivered to the target is measured by the following parameters: Energy (Joules = Watt x Seconds), Energy Density (Joules/cm^2), Irradiation Time (Seconds), and Spot Size (The area of the spot the laser light illuminates). The dose administered in this study were calculated as described in the materials and methods section.

Figure 2. Conventional Optical Resonance Laser

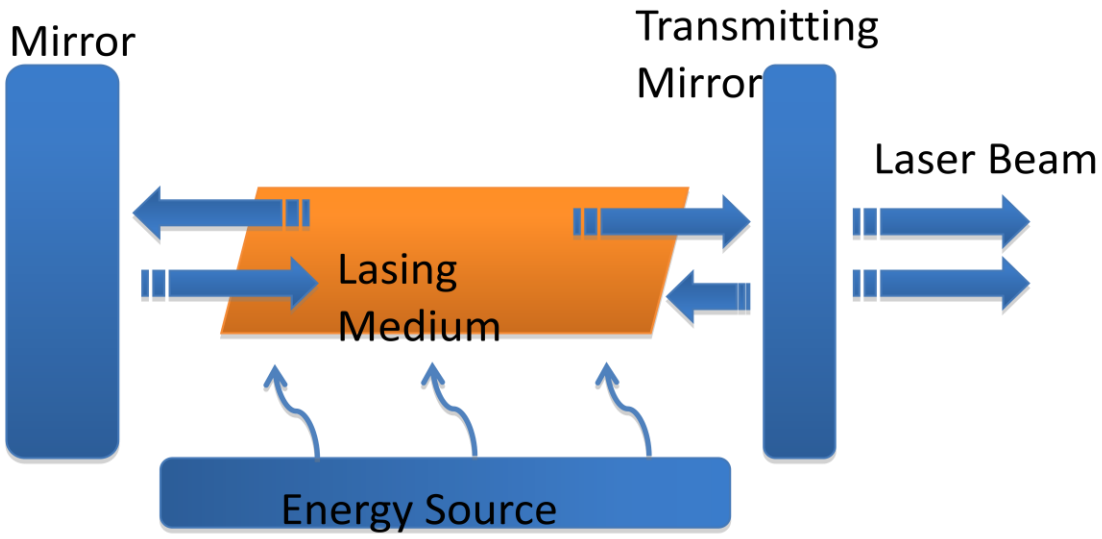
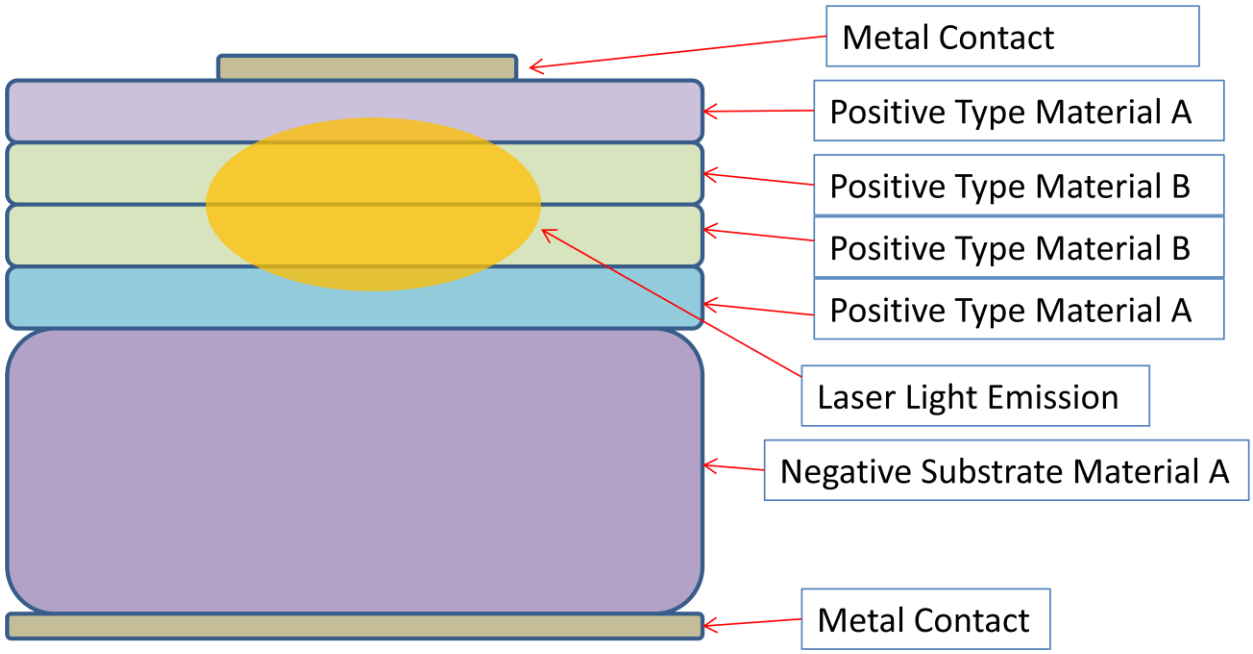


Figure 2 shows basic principles, in a conventional optical resonance laser, required to create laser light. The lasing medium is placed between two mirrors. One of the mirrors is completely reflective. The transmitting mirror allows some of the laser light to escape creating the laser beam. An energy source is required to start and sustain the laser beam.[18]

Figure 3. Diode Laser (Like the GaAlAs laser used in this experiment)



In a diode laser, as used in this study, the laser beam is generated from a semi-conductor diode. The laser light emitted in a diode laser is similar to the conventional optic laser light as it is created by exciting electrons and providing an energy hole where the electron can fall allowing it to release energy in the form of laser light. [18]

1.4 Low Level Lasers

Low level laser irradiation (LLLI) is the application of low power laser light (1mw-500mw) commonly used to increase tissue regeneration, decrease pain, or to reduce inflammation. It is usually in the near infrared spectrum (800nm-1000nm), with an incident power between 1mw to 500mw/cm^2. LLLI does not function by heat or vaporization rather it produces a photochemical effect much like plant cells do to sustain life.[19] When the laser light encounters an object that can absorb the photon, the energy from the photon is not destroyed but used to increase the energy level of the absorbing atom or molecule.[18] The first law of photobiology, the Grotthus-Draper Law[20], explains that if LLLI influences the biological system the photons must be absorbed by electron absorption bands that belong to photoacceptors or chromophores.[19] These chromophores can be seen in cytochrome c oxidase[21] hemoglobin, melanin, myoglobin, flavins, and flavoproteins[19]. It is believed cytochrome c oxidase in the mitochondria is the principle chromophore responsible for the effects seen from LLLI[19]. The LLLI in this study is assumed to affect the human osteoblast at the mitochondria creating an environment within the cell to increase or decrease cell proliferation and alkaline phosphatase production. The effect seen on biological specimens has been reported is known as the Arndt-Shulz law governing cellular response to applied energy. The Arndt-Shulz law governing cellular response to applied energy **s**tates that weak stimuli increase vital activity, even greater stimuli will raise the vital activity further until a peak is reached as seen in Figure 4. Once this peak has been reached, the increases in further stimuli suppress the vital activity. [19]

Figure 4. Diagram of the Dose/Response Curve Depicting the Arndt Shulz Law

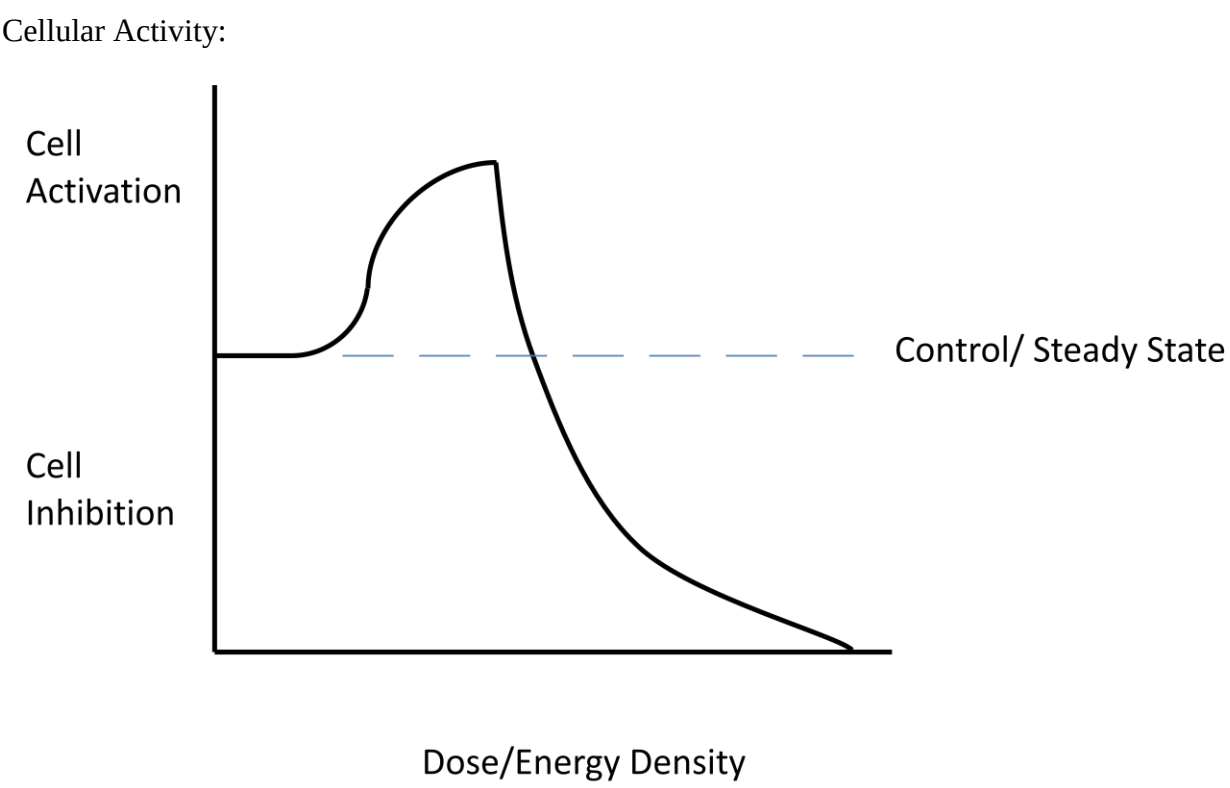


Figure 4 demonstrates graphically the Arndt Shulz Law. The Arndt Shulz law states that weak stimuli (low dose) increase vital activity, even greater stimuli will raise the vital activity further until a peak is reached. Once this peak has been reached stronger stimuli suppress the vital activity. [19]

1.4.1 History of Low Level Lasers

In 1400 B.C. Indians were treating vitiligos using a lotion with a plant preparation called psoralens and then exposing the patients to sunlight. The Egyptians later used a similar technique to treat leukoderma.[18] Nearing the end of the 19th century Dr. N.R. Finsen, the father of contemporary phototherapy, was treating dermal tuberculosis, smallpox, and measles with ultraviolet and red light.[20] In 1974, psoralens combined with ultraviolet A radiation was recognized as an effective treatment for psoriasis and vitiligo. These early uses of light to help cure illness demonstrate that light does indeed have curative effects on human organisms. The reader may more fully appreciate the effects of light when he or she remembers the last time overexposure of the sun light caused the skin to react creating a sun burn or ponders upon the thousands of cases of skin cancer thought to be in part a result from over exposure to sun light.

The first laser (a ruby laser) was developed by Theodore H. Maiman of Hughes Aircraft Corporation in 1960. In 1967 low level laser therapy began to emerge when Endre Mester in Budapest, Hungary was irradiating shaven mice to evaluate the effects of lasers on skin cancer. He found hair grew back quicker on the treated groups of mice.[22] Today the term low level laser irradiation is used to differentiate it from the higher power ablative lasers that are also used in medicine.[20]

1.4.2 Low Level Laser Therapy in Dentistry and Orthodontics

Multiple areas in the medical field are being tested with low level leser therapy. Some promising results in the dental field are found when LLLT is used with: burning mouth syndrome[23], chronic gingivitis[24], rapid maxillary expansion[25], alveolar osteitis[26], healing extraction sockets in diabetics[27], decreasing pain during cavity preparations on children[28], increasing orthodontic tooth movement[29], dentinal hypersensitivity[30], and managing myofascial pain[31].

Low-Level Laser Therapy (LLLT) is beginning to emerge as a technology that may decrease Orthodontic treatment time[7]. It is believed that LLLI affects the proliferation of osteoblast cells that build bone but it is not yet clearly understood how the laser acts on the cells[10]. Increased activity of these bone remodeling cells may allow faster tooth movement while decreasing or inhibiting these cells could decrease tooth movement. Controlling the rate of tooth movement through the alveolus during orthodontic treatment would enhance treatment time.

1.4.3 Significance of Low Level Laser Therapy in Orthodontics

One of the major concerns in orthodontic tooth movement is prolonged treatment time. LLLT emerged as a viable technology to manipulate tooth movement during orthodontic treatment. To date, limited literature is available on the effects of Low-Level Lasers on orthodontic movement in humans. Cruz et al in their studies with humans used a GaAlAs laser at 780nm, irradiation for 10 seconds at 20 mw, with 5 J/cm^2 on 4 days of each month. They found that LLLT accelerated human canine retraction movement by 1mm than the control after 60 days of treatment [7]. In another similar study, 860 nm of GaAlAs laser with power output 100 mW, spectral area 0.09

cm^2, power density 1.11 W/cm^2, energy dose 2.3 J/point and energy density 25 J/cm^2/site with continuous mode was used to irradiate the alveolar mucosa. The laser irradiation was given at three points on buccal and palatal sides, and two points at the distal of the canine with 23 seconds per point[32]. In this study it was discovered that the means of distal movement between the irradiated side and the placebo side had no significant difference for any time periods evaluated and attributed this to the belief that they did not have enough surface energy density with their GaAlAs laser of 25 J/cm^2 [32]. Another study with rat model proved that LLLI accelerated the velocity of tooth movement by stimulating alveolar bone remodeling[33].

Another concern in orthodontic tooth movement is pulpal tissue damage during increased tooth velocity. LLLI offers a quicker repair of pulpal tissue due to orthodontic tooth movement[34]. This makes LLLT an exciting proponent for increased pulpal health of orthodontic patients.

Another orthodontic benefit of LLLT is that at the optimum dosage it may diminish tooth movement[35]. Increased orthodontic anchorage can also decrease orthodontic treatment times.

The above studies indicate LLLI may have the capacity to decrease or increase tooth movement during orthodontic treatment. However, there is no information available on optimum dose response on human osteoblast cells.

Fortuitously, this research can also shed light on other areas of interest outside of orthodontics. The cells used for this study are MG-63 osteosarcoma cells (malignant osteoblasts). Tuner and Hode explained that LLLT could be a viable option for pain control and general stimulation for cancer or suspected cancer patients [36]. Tuner and Hode stated that LLLT as a radioprotective effect on tissue [36]. Da Cunha performed LLLT on rats that were submitted to radiotherapy and showed that it improved bone density, increased bone marrow cells, and

increased osteoblastic activity.[37] Zimin et al. showed that LLLT does not cause formation of neoplasms but there is caution used because of the possible stimulatory effect on tumor growth. In Zimin's article the results of LLL treatment on patients with oncological diseases spanning the last 25 years were evaluated. He found that at 2-4 year follow up observations no increase in the frequency of metastasis and tumor recurrence were detected. [38]

1.5 Purpose

The purpose of this study is to evaluate cell proliferation, and alkaline phosphatase production of immortalized human osteoblasts in response to low-level laser irradiation (GaAlAs 830nm). In addition, the findings are analyzed and compared with the Arndt-Shulz Law governing cellular response to applied energy on cultured human osteoblasts. The Arndt-Shulz law indicates that weak stimuli excite physiological activity and strong stimuli retard it. The implication of LLLI on osteosarcoma cancer is also discussed. The objective is to evaluate applicability of the Arndt-Shulz law by investigating the dose of laser energy that inhibit or enhance human osteoblasts activity as monitored by the assessment of cell proliferation, viability, and alkaline phosphatase activity. The findings will also reveal further information on the potential for LLLI to influence tooth movement in orthodontics.

This *in vitro* study of human osteoblasts and LLLI, and may be looked at as a pioneering step toward a dose response curve for osteoblast activity that may lead to significant enhancement to modern day orthodontic biomechanics and further understanding of the effects of LLL treatment on osteosarcoma. Admittedly, another study performed similar to this one with human osteoclasts would shed more light on the capacity LLLT may provide for the future of

orthodontic tooth movement. Once the dosage to up-regulate and down-regulate human osteoblasts and osteoclasts is known the research can move toward human experimentation with a greater understanding and precision. The exact dose necessary to cause the desired biomechanical response may be illuminated.

This research relates clinically to orthodontics because the capacity to regulate the osteoblast cell activity may lead to decreased treatment time and increase orthodontists' productivity enhancing well being for all. This research points to a day when orthodontic braces are perhaps mounted with fiber-optics administering correct doses of LLLI to the alveolus encased root and periodontium stimulating or inhibiting orthodontic tooth movement to the orthodontists' desire. The outcome of this project will provide basic information for the application of LLLI in orthodontics.

1.6 Specific Aims and Hypotheses

1.6.1 Specific Aim 1: Dose Response Curve

A. To measure and compare human osteoblast cell viability and proliferation at 24, 48, 72 hours time period after administering a range of low to high doses of LLLI using 20mw, time exposures ranging from 6 to 90 seconds, a spot size of 0.252 cm^2 and energy densities ranging from 0.4-6.01 J/cm^2 in contact mode.

1.6.2 Specific Aim 2: Alkaline Phosphatase

B. To determine any differences in Alkaline Phosphatase activity at 10 days.

1.6.3 Hypothesis

The central hypothesis to be investigated is that at low doses of LLLI human osteoblast activity increases, and at high doses activity decreases. In other words, an Arndt-Shulz type dose response curve should result. Obtaining this dose response curve may help decrease orthodontic treatment time in the future and may shed light on contra-indications of use of LLLI. Human osteoblast activity will be defined by cell viability, proliferation, and alkaline phosphatase production. To test this hypothesis a range of doses from low to high will be compared. We expect greater human osteoblast activity with lower doses of LLLI and decreased osteoblast activity at higher doses; generating a type of dose response curve. It is also hypothesized that caution should be used when applying LLLI to patients with cancer because of possible up regulation of the cancer cells as seen *in vitro*.

1.7 Location of Study

This study was conducted in the Craniofacial Research Center Room # 7391, College of Dental Medicine, 3200 South University Drive, Nova Southeastern University, Fort Lauderdale, FL 33328.

Chapter 2: Materials and Methods

2.1 Research Design

In this study human osteoblast like cells derived from osteosarcoma cultures were used. Cells were grown in complete medium until it reached 70-80% confluency. The cells were then dislodged and suspended in the growth medium. Based on experimental plan the cells were plated in either 96 well plates (as seen in figure 5) or 12 well plates. Experiments on cells proliferation and osteogenic differentiation were performed. The cells plated in 96 well or 12 well plates were exposed to low level laser irradiation at specific duration of exposure with different energy densities and doses. The cell proliferation and alkaline phosphatase activity was measured at designated time points. Cell proliferation assay was carried out in a 96 well plate at 1, 2 and 3 day intervals. These time points were selected because effect of lasers on cellular activity was observed as early as 24 hours in mouse osteoblasts cells.[48]

Differentiation is another hall mark phenomenon that may be induced by low level lasers. Alkaline phosphatase is an important biomarker for osteogenic differentiation. Although many studies have reported the positive effect of low level lasers on osteogenic differentiation in many types of cells[39] no study has indicated the optimum dose for favorable osteogenic differentiation. In this study activity of alkaline phosphatase was measured at different time points. The cells without irradiation were considered as control group and cells. Our pilot study demonstrated that there were no measurable levels of alkaline phosphatase produced at any dose after 1, 2, 3 and 5 days on cell differentiation The activity of alkaline phosphatase was observed at 10 day post irradiation. The dose and time of irradaiation was determined based on our pilot experiment. The cells cultured in complete medium or osteogenic medium were exposed to laser and activity of alkaline phosphatase was measured at 10^{th} day post treatment.

The cells were irradiated by the GaAlAs laser in contact mode for the cells plated in 96 well plate. Figure 6 demonstrates contact mode with the laser in contact with the bottom of the 96 well culture plate. The cells plated in 12 well plate to carry out ALP assay cells were irradiated by holding the Asah 450 probe at a distance of 72 inches from the cells allowing the GaAlAs laser to diverge to nearly completely irradiate the area of one well in the 12 well plate as seen in figure 7. A black piece of cardboard paper was placed over the 11 wells not to be exposed allowing only the desired cell well to be irradiated. The detailed methods of cell culture and irradiation methods, dose determination and experimental procedures are given in detail in the following sections.

Figure 5. Plating of Osteosarcoma Cells

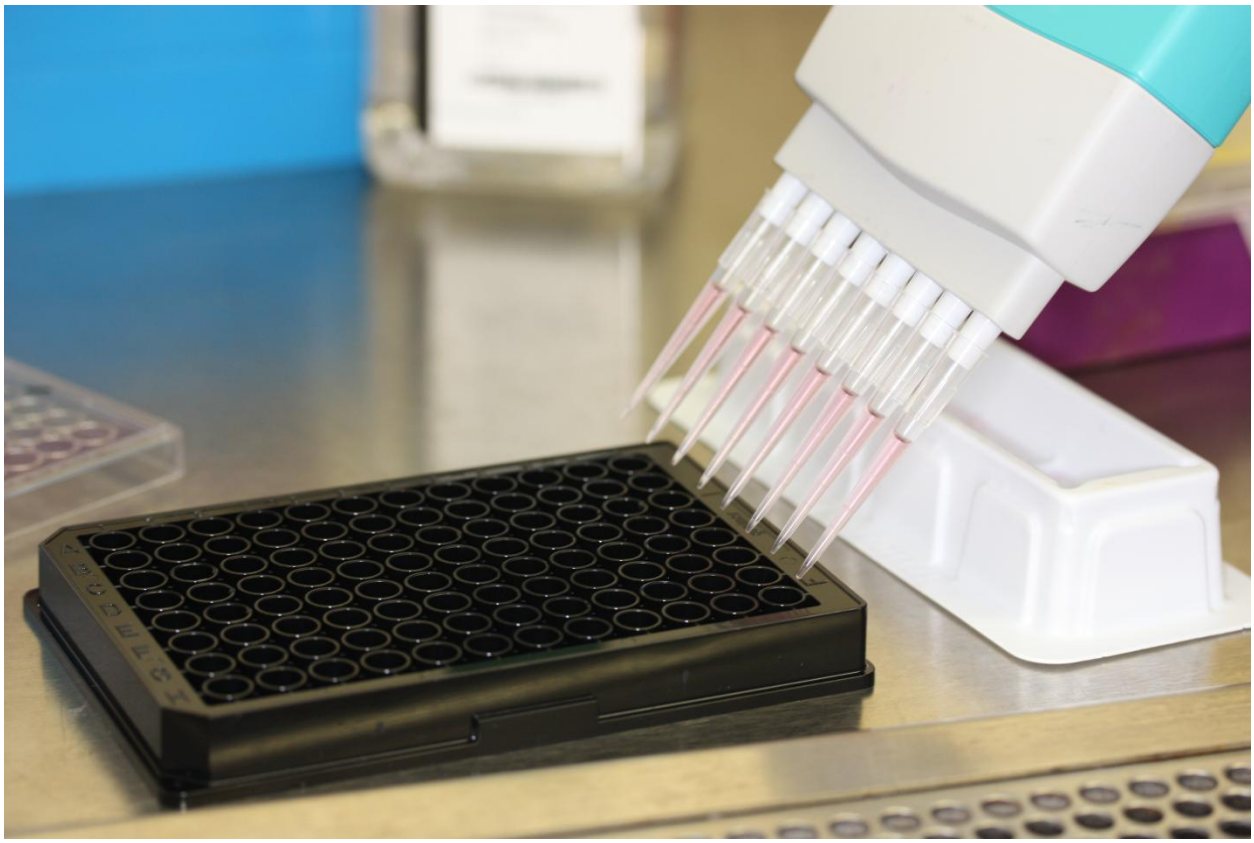
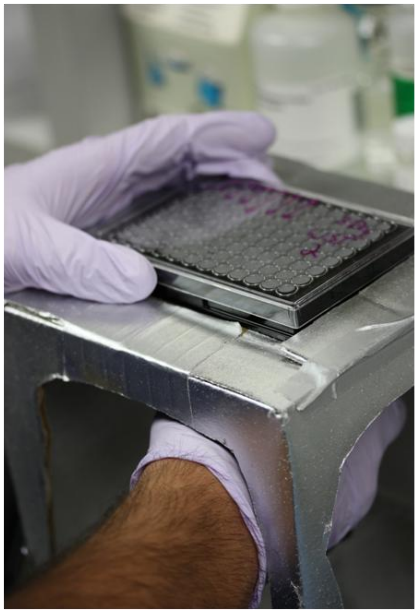


Figure 5 depicts the plating of osteosarcoma cells in 96 well plates. The cells were suspended in complete medium or growth medium with 5,000 cells per 100 uL in each well. Notice the use of a multi-pipette dispenser for maximum accuracy.

Figure 6. Cell Irradiation in Contact Mode

A

B **C** **D**



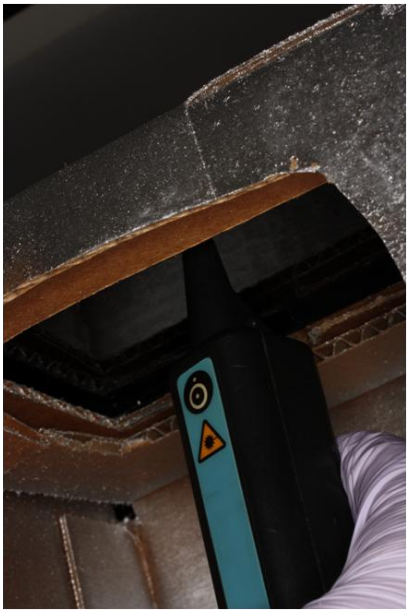


Figure 6 demonstrates: A. Osteoblasts inside the wells in which they will be irradiated with the laser light passing through the bottom of the well into the cells. B. C. & D.: Demonstrate the actual procedure. The 96 well plates were placed on top of the lasing apparatus with a cut out allowing the laser to be in contact with the bottom of the wells. The Asah 450 stylus (or probe) was then held below the wells and the cells were irradiated.

Figure 7. LLL Irradiation Setup for 12 well plate

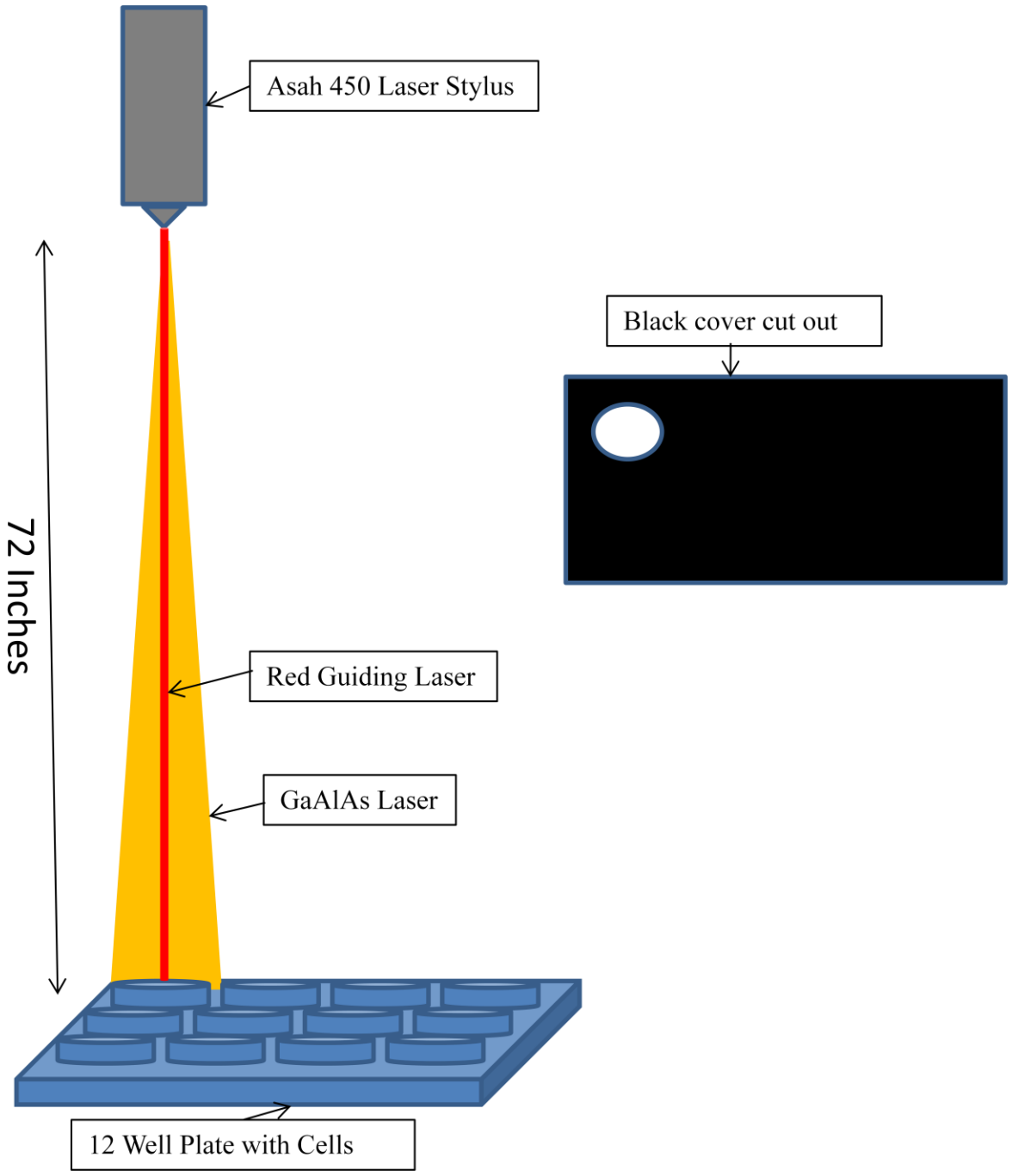


Figure 7 shows the Asah 450 laser stylus held at 72 inches from the cells in the 12 well plate. A black cover cut out was positioned over the top of the 12 well plate to allow exposure of only 1 well and to inhibit cross contamination to the other wells.

2.2 Cell Culture

Human osteoblast–like cell line MG-63 was obtained from the American Type Culture Collection (ATCC# CRL 1427**,** Manassas, VA 20110). MG -63 cells were derived from a human osteogenic sarcoma and possess an osteoblastic phenotype[40, 41]. Cells were grown in Minimum Essential Eagle's medium (MEM; ATCC# CRL 1427**,** Manassas, VA 20110) supplemented with 10% fetal bovine serum (Atlanta Biologics, Lawrenceville, GA, USA) and 1% antibiotic and antimicotic solution. The cells were fed with a fresh MEM every 2-3 days. Cultures were propagated at 37 °C under humidified conditions using 5% CO_2. The cells grown up to 70 – 80% confluent were treated with trypsin to dislodge from the flask. Suspended cells were plated either in 96 well plate or 12 well plate based on the experimental design and experiments were conducted to investigate the effects of LLLT on osteosarcoma cell viability and differentiation.

2.3 Low Level Laser Irradiation (LLLI)

LLLI was performed using the Asah 450 GaAlAs (Asah Medico, Hvidovre Denmark) at wavelength of 830 nm, with a visible guiding light laser that is 670 nm as shown in figure 8.

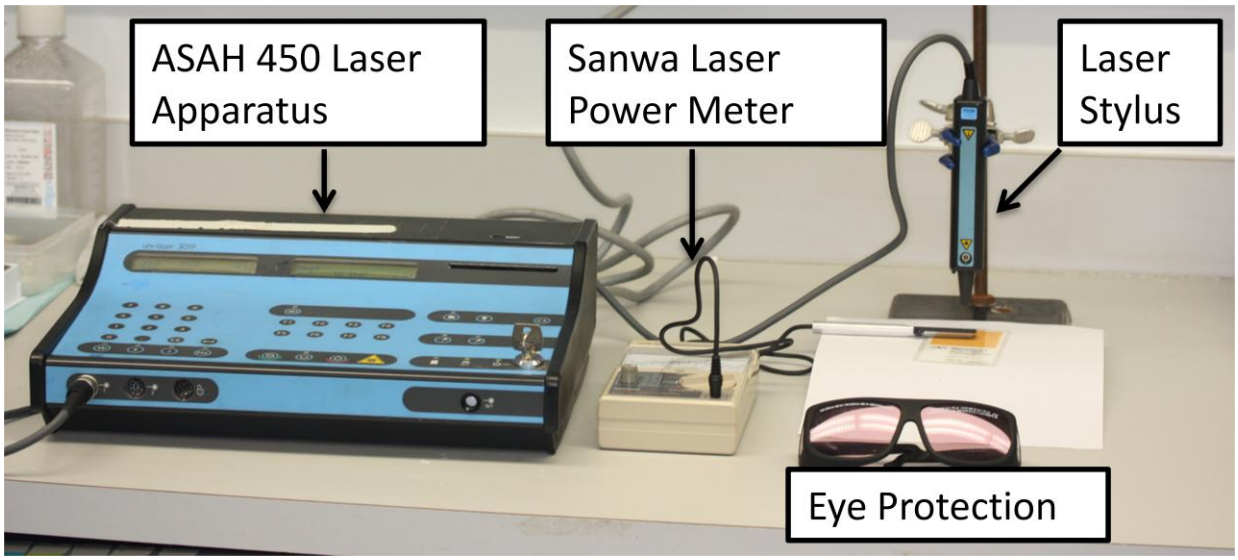
The laser specifications for the stylus of the ASAH 450 indicate that it irradiates a spot size of 0.1 cm^2. In order to verify that this spot size is correct an infrared sensor card (Newport Model F-IRC1 Irvine, Ca.) was obtained to visualize and measure the actual spot size of the GaAlAs laser. When the authors measured the actual spot size from the lasers origin was determined to be 0.252 cm^2. The actual area of the bottom of the 96 well plate is 0.317 cm^2 according to the manufacturer as shown in figure 9.

The 0.252 cm^2 spot size was believed to be circular but was impossible to get a precise reading because the red guide laser does not turn off during the GaAlAs irradiation. A green

filter was then used but was not able to reduce the red guiding laser enough to obtain an unbiased reading of the actual spot size. When the GaAlAs laser is fired it glows a bright yellow on the infrared sensor card. The red guide light remained red. The authors decided to move the laser to a distance of 72 inches and examined the divergence of the GaAlAs laser in comparison to the red guiding laser. At this distance the red guiding laser remained about the same spot size but the GaAlAs laser experienced much greater divergence reaching a spot size of about 11mm x 23mm. The sensor card showed that the GaAlAs laser actually has a rectangular spot size with areas of higher and lower intensity forming a kind of grid as seen in figure 10.

The Asah 450 Laser was sent to its origin company for evaluation of function and calibration. A Sanwa Laser Power Meter (Sanwa Electric Instrument Co., Ltd. Tokyo, Japan Model OPM-0572) was used to further evaluate calibration as seen in figure 11. This laser detector is capable of measuring wavelengths in the 830nm range, power range of .01 to 30mW with an accuracy of $\pm$5% of full scale reference wavelength. It was determined that when the laser was set to 20mW the laser power meter averaged 19mW after three exposures.

Figure 8. Asah 450 and Laser Equipment



Starting at left the Asah 450 laser apparatus is shown in figure 8. The apparatus was used to input the proper irradiating parameters including mW's and time of exposure. At center, the Sanwa laser power meter was used to calibrate the Asah 450 laser and to calculate any attenuation that the laser light may experience as it passes through the air and plastic bottom of the 96 cell well plate. Next, the eye protection is shown and was used during sessions of irradiation for safety. At right, the laser stylus is shown and was calibrated in this orientation.

Figure 9. Spot Size Calculation

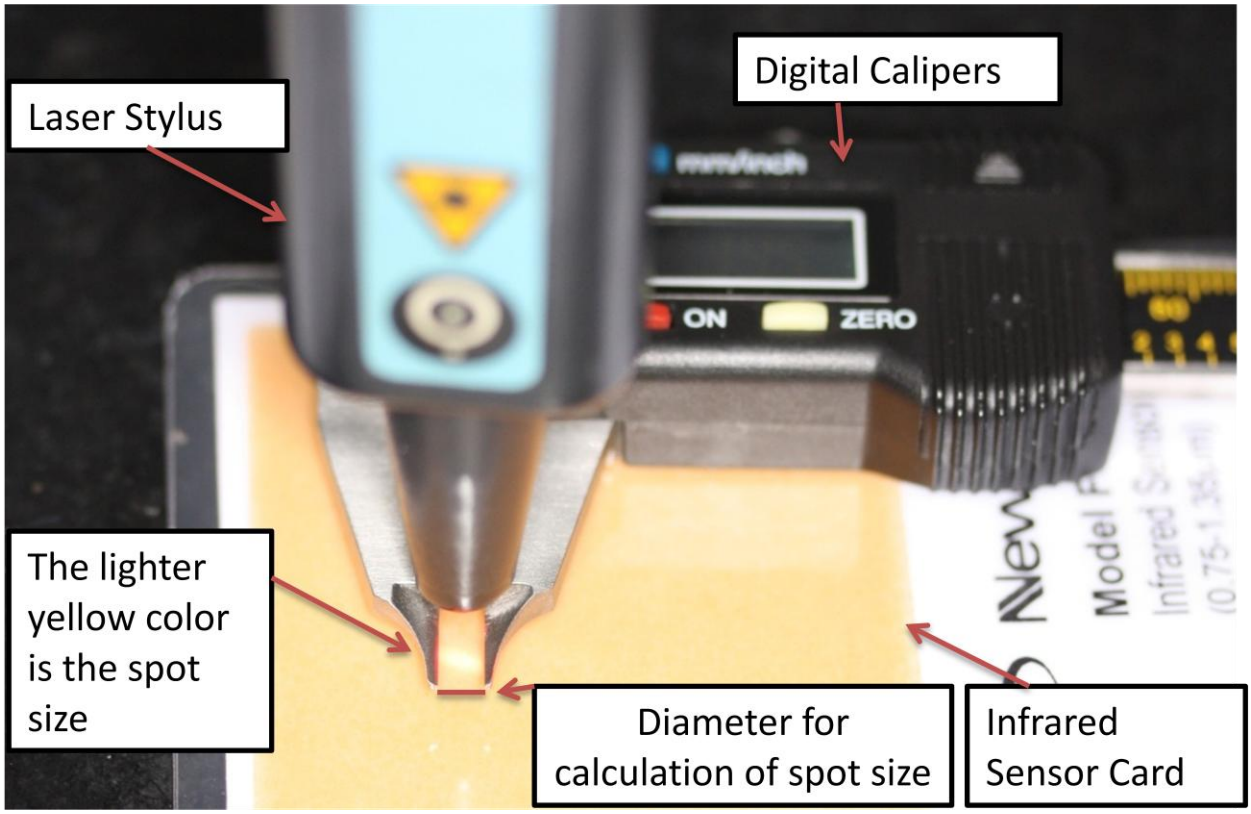


Figure 9 demonstrates how the laser specifications for the probe of the ASAH 450 were analyzed. Some difficulty was found in calculating the actual spot size even with the sensor card as the visible red laser light remains illuminated during the exposure with the GaAlAs laser light. A green filter (not shown here) was placed just below the stylus in order to inhibit the confounding visible red guiding light in order to better evaluate the spot size from the GaAlAs laser light. The digital calipers were placed just above the infrared sensor card. The Asah 450 was then irradiated for an extended period of time in nearly contact mode so that the authors could measure the diameter of the spot as outlined on the infrared sensor card. The authors best efforts determined the actual spot size from the lasers origin was .252 cm^2. The actual area of the bottom of the 96 well plate is .317 cm^2.

Figure 10. Divergence and Grid of GaAlAs Laser

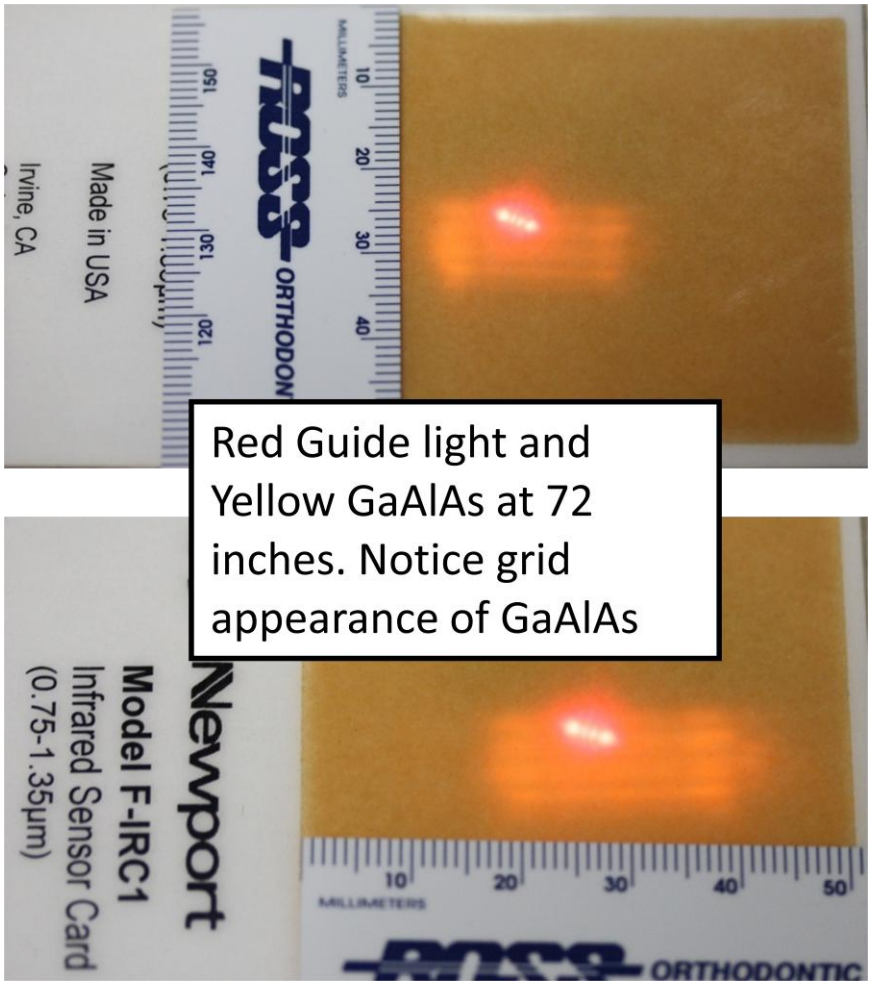


Figure 10 shows the divergence of laser irradiation.The grid pattern that is experienced by the GaAlAs laser at 72 inches from the tip of the stylus. The visible red guiding laser light can be seen at the top left of the illuminated rectangular orange appearance of the infrared GaAlAs laser light. The GaAlAs laser light appears to illuminate in a rectangular shape with a grid pattern. The spot size was calculated by measuring the illuminated rectangle.

Figure 11. Sanwa Laser Power Meter

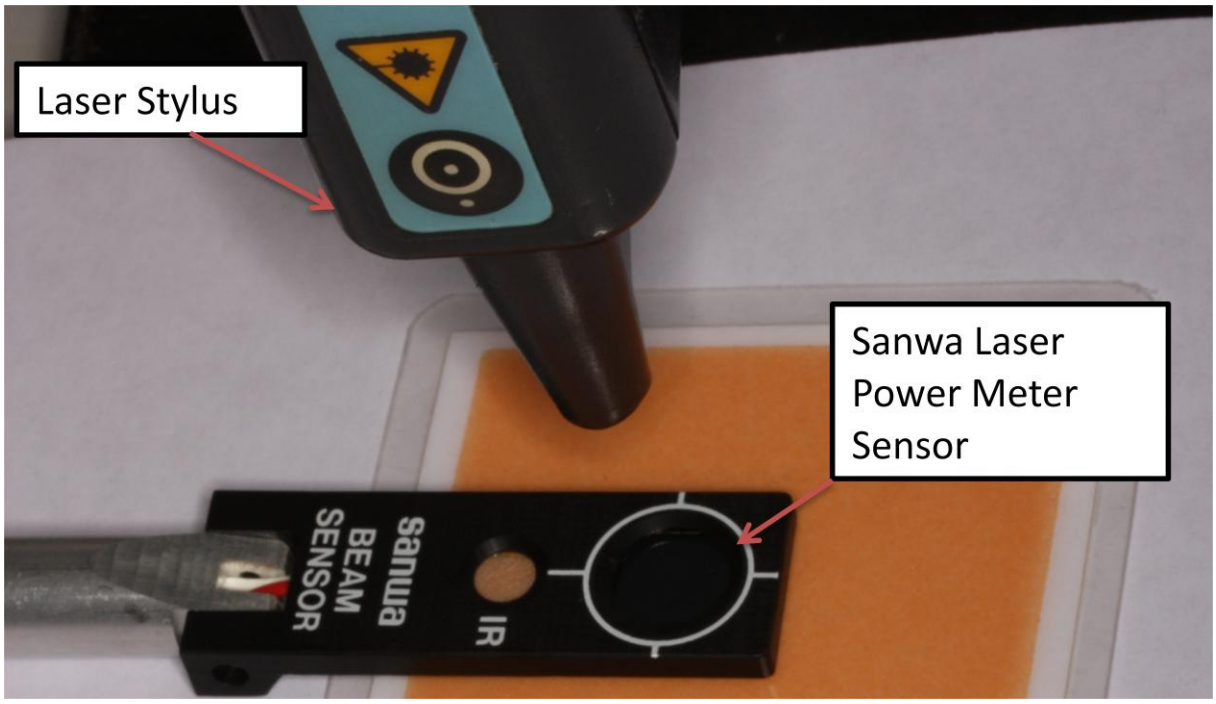


Figure 11 shows the laser stylus being lowered to be nearly in contact with the Sanwa laser power meter in order to calibrate the Asah 450 laser. When the Asah 450 apparatus stated that it was to produce 20mW we found that it averaged 19mW.

Owing to the fact that the laser light would pass through the bottom of the 96 well plate the laser light was checked for attenuation. This was done by removing the clear bottom of one of the 96 wells and irradiating through it to our laser power meter as seen in figures 12 and 13. After three exposures, with the laser light set at 20 mW on the Asah 450, through a cut out well bottom (measured to be 0.27mm thick) an average of 16.83 mW would be reaching the cells at the bottom of our 96 well plates.

The black 96 well plate with a clear bottom was chosen in order to decrease or eliminate any cross exposure (contamination) of the laser light from one well to the next. In order to verify that no laser light would pass through the black well walls, a piece was cut from the plate and evaluated. 0 mW were recorded after three trials of lazing through the black well walls (.8mm thick) as seen in figure 14.

Figure 12. Bottom Cut Out of 96 Well Plate to Check for Attenuation

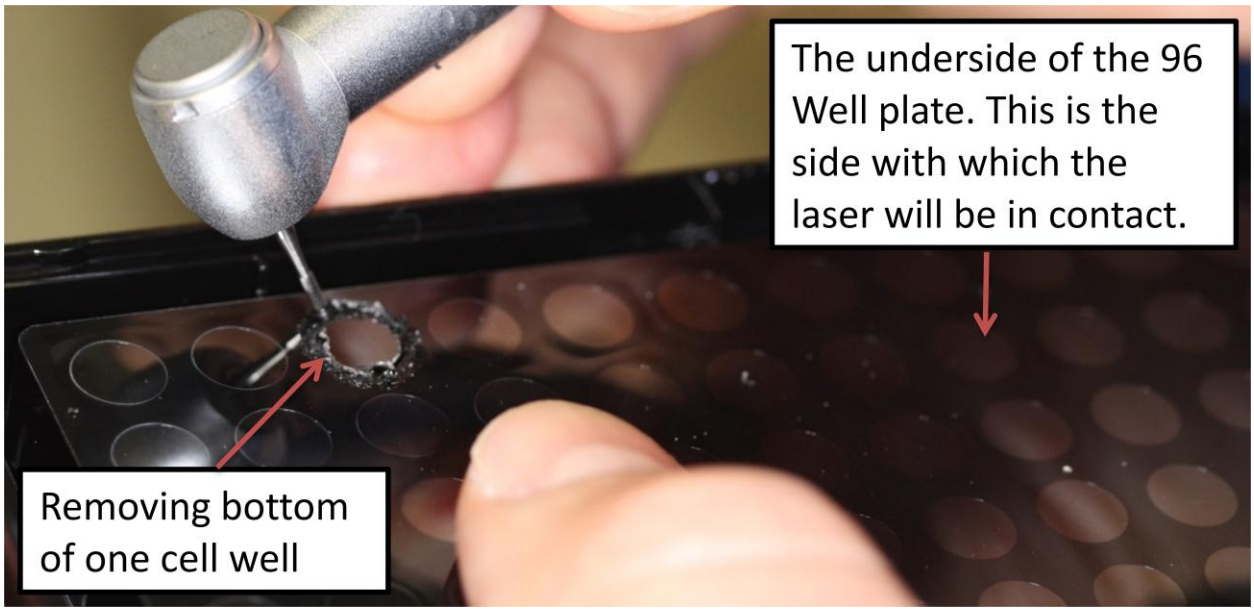


Figure 12 demonstrates the bottom view of the 96 well plates that were used for irradiating the osteoblasts. In this figure a dental handpiece with a carbide bur was used to remove the bottom of the well in order to measure its thickness and to determine the amount of attenuation that the laser light experiences before it reaches the osteoblasts that will be residing on its surface. The laser stylus will be in contact with the bottom side of these wells in what is described as “contact mode”. Note that between each of the wells there is a layer of black plastic that will inhibit cross contamination of laser light during exposures.

Figure 13. 96 Well Bottom Cut Out and Placed Over Laser Power Meter for Attenuation

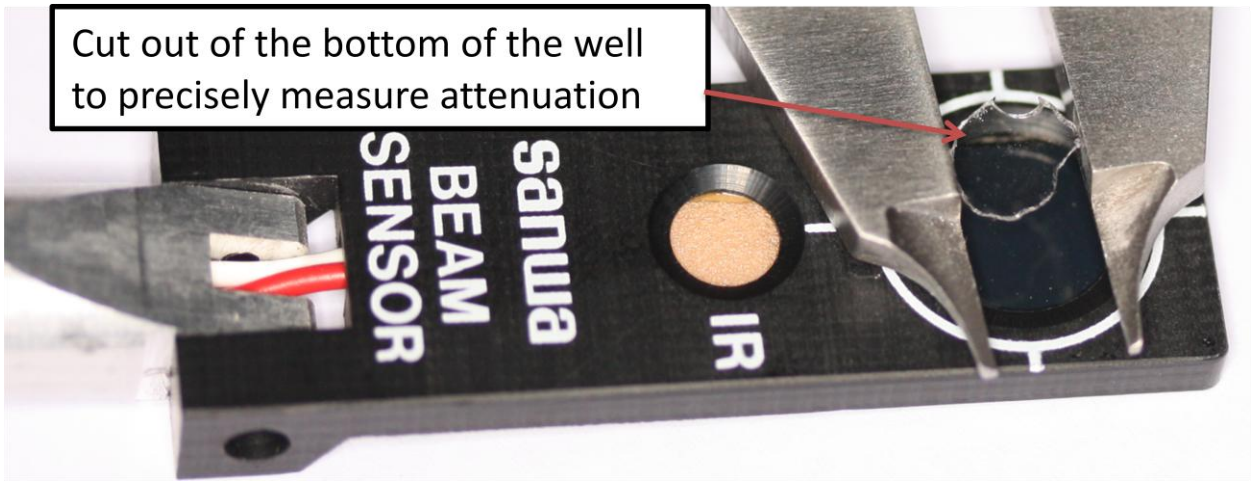


Figure 13 demonstrates how the cut out bottom of the well was held by digital calipers over the Sanwa laser power meter. The laser light was passed through the cut out piece in contact mode. The Sanwa beam sensor was placed just below the well (identical to where the osteoblasts would reside in relationship to the laser and the cell well bottom). Attenuation was determined in this manner. It was determined that when the Asah 450 apparatus stated it was producing 20mW the osteoblasts would experience 16.83mW due to attenuation of the bottom of the cell well.

Figure 14. Black Side Wall of 96 Well Plate

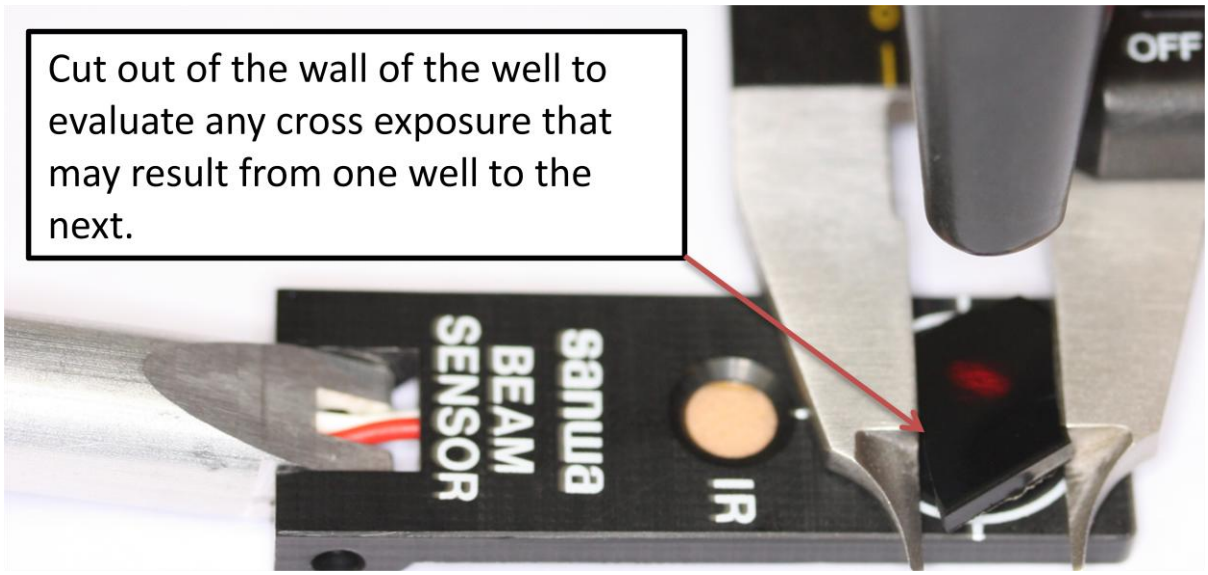


Figure 14 demonstrates how the cut out black walls of the 96 well plate was held by digital calipers over the Sanwa laser power meter. The laser light was passed through the cut out piece in contact mode. (This figure shows the laser light reflecting off the black piece of well wall and is not in contact mode. The actual testing was done in contact mode meaning the stylus was in contact with the plastic wall).The Sanwa beam sensor was placed just below the well to determine the amount of attenuation and elimination of cross contamination that should be expected during irradiation of the osteoblasts. It was determined that when the Asah 450 apparatus stated it was producing 20mW there would be 0mW passing through the well walls completely eliminating cross contamination of laser light.

2.3.1 Determination of Dose:

Dose determination for 24, 48 and 72 Hours WST Assays:

A search for optimal proliferative and inhibitory doses (Appendix A) was made from a diligent literature review. In accordance with the above literature and the desire to find both proliferative and inhibitory doses it was determined that a range of laser doses would be given in contact mode, continuous pulse, and no booster doses, as noted in Table 1.

The format with which these dosages were administered to the 96 well plates is shown in figure 15.

Dose Determination for ALP Assays:

No measurable quantities of alkaline phosphatase were observed at 24, 48, 72, and 120 hours in our pilot study for the ALP assays. It was determined that at 10 days and in a 12 well plate measurable quantities could be observed. The author utilized the information from the proliferative doses acquired performing the WST assay and replicated interesting doses, as closely as possible, in a 12 well plate (Table 2) with the laser stylus at 72 inches from the plate as seen in figures 16 and 7. The Sanwa Laser Detector was used to evaluate the energy density experienced by the cells at the new distance.

Table 1. Laser Irradiation Doses: 96 Well Plate Treatment

Cell Column	Machine mW	Actual mW Reaching	Time Exposed (S)	Joules Produced	ActuSpot Size (cm^2)	Energy Density(J/cm^2)
1	20	16.83	6	0.10098	0.252	0.401
2	20	16.83	12	0.20196	0.252	0.801
3	20	16.83	18	0.30294	0.252	1.202
4	20	16.83	24	0.40392	0.252	1.603
5	20	16.83	30	0.5049	0.252	2.004
6	20	16.83	36	0.60588	0.252	2.404
7-12	Control	0	0	0	N/A	0.000

Cell Column	Machine mW	Actual mW Reaching	Time Exposed (S)	Joules Produced	ActuSpot Size (cm^2)	Energy Density(J/cm^2)
4	20	16.83	60	1.0098	0.252	4.007
5	20	16.83	75	1.26225	0.252	5.009
6	20	16.83	90	1.5147	0.252	6.011
7-12	Control	0	0	0	N/A	0.000

Table 1 demonstrates the parameters used to calculate the energy density created during irradiation in contact mode. Notice that the Asah 450 apparatus stated 20mW but the actual mW's reaching the cells at the bottom of the cell wells is 16.83mW due to attenuation. Joules produced during the exposure are noted.

Figure 15. Dose Layout for 24, 48, and 72 Hour Experiments

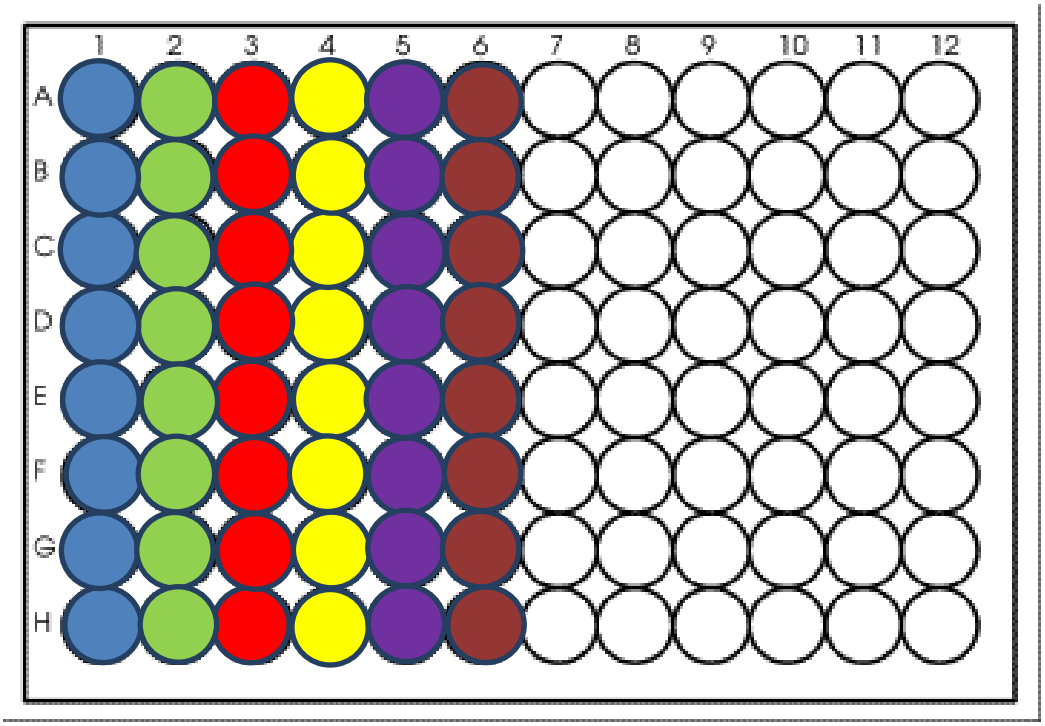


Figure 15 demonstrates the dose layout as it was administered in the 96 well plates for time periods 24, 48, and 72 hours. Column 1 has 8 wells and each was exposed with 6 seconds creating an energy density of 0.40 J/cm^2 in each well. Each succeeding column was irradiated accordingly and columns 7-12 which were used as controls. The 60, 75 and 90 second exposures were performed in a similar layout.

Table 2. Laser Irradiation Doses: 12 well plate

Machine mW	Actual mW Applied	Time Exposed in seconds	Joules Produced	Actual Spot Size (cm^2)	Energy Density(J/cm^2)
85	85	18	1.53	1.76	0.8693
85	85	48	4.08	1.76	2.3182
269	269	18	4.842	1.76	2.7511
269	269	48	12.912	1.76	7.3364
269	269	60	16.14	1.76	9.1705
Control	0	0	0	N/A	0

The author utilized the information from the proliferative doses from the MTT assay and replicated the interesting doses, as closely as possible, in a 12 well plate as indicated above.

Figure 16. Dose Layout for ALP assay experiment

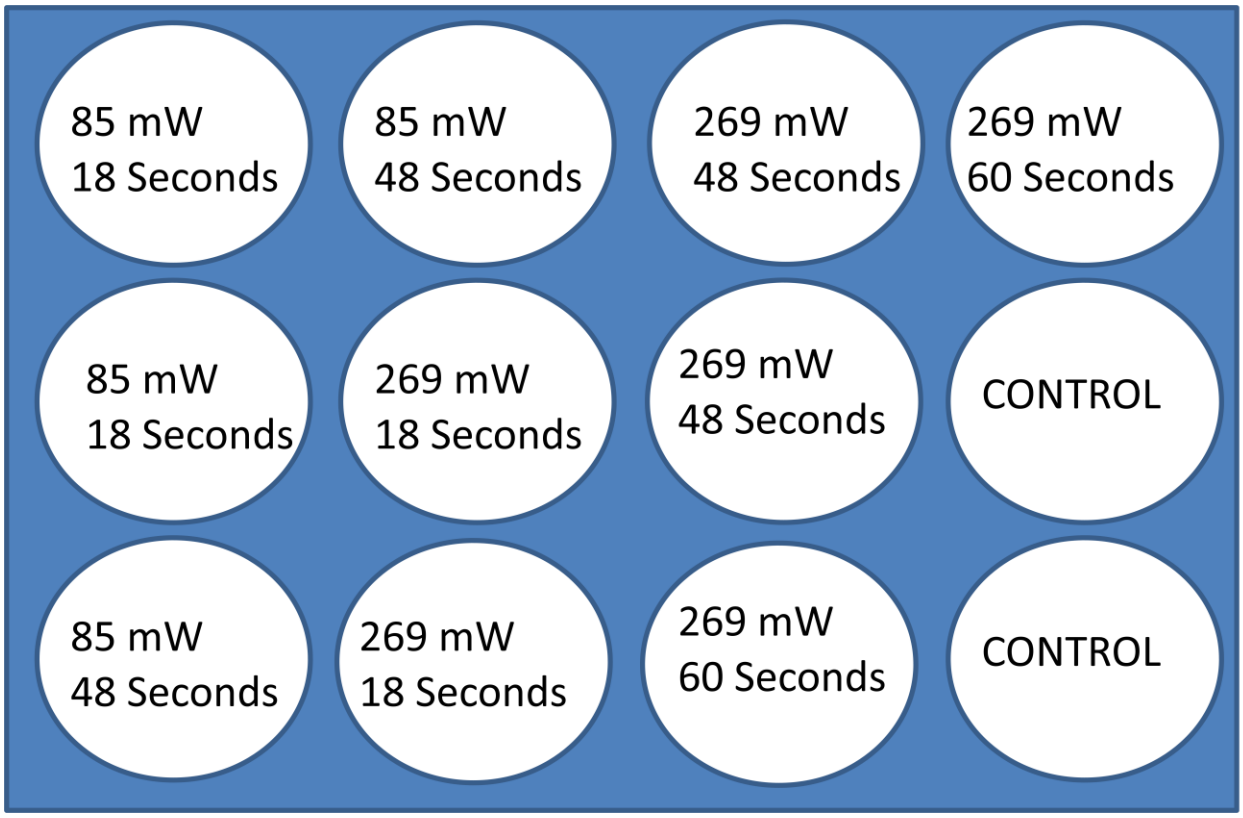


Figure demonstrates the format in which the osteoblasts were placed within the 12 well plates for irradiation.

2.4 Cell Proliferation Assay (WST-1 Assay)

The cells dislodged from T 75 flaks were suspended in growth medium. Approximately 5,000 cells per 100 μL were seeded in each well of the 96 well plates and allowed to adhere for 3-4 hours. The cells were then irradiated by the method described above and were returned to the incubator and incubated at 37°C with 5% CO2 for 24, 48, and 72 hour time intervals. After each designated time intervals the medium was removed and cells were washed twice with phosphate buffered saline (PBS) and cell proliferation assay was performed based on the cleavage of tetrazolium salt WST-1 (Roche Diagnostic GmbH, Lot 12417300, Manheim, Germany) by mitochondrial dehydrogenases in the viable cells. Briefly, WST-1 reagent was added to each well at 1:10 ratio to cell culture medium. After 4 hours of incubation in a humidified atmosphere with 5% CO^2 at 37°C and absorbance was measured at 490 nm using a microtiter plate reader (DTX 880 multimode detector; Beckman Coulter, Fullerton, CA, USA) at 450 nM (figure 17). The cells without irradiation were considered as controls the cell viability of experimental group was compared to control group. The percent viability was measured as:

i.e Viability= (Absorbance of laser treated sample/ Absorbance of control) X 100

Figure 17. Microplate Reader

Figure shows the microplate reader (DTX 880 multimode detector; Beckman Coulter, Fullerton, CA, USA) that was used to measure the absorbance values for cell proliferation assay and ALP activity assay.

2.5 ALP Assay (pNPP Method)

Cells suspended in culture medium were plated in 12 well plates with a density of $1x10^3$. After removal of culture medium, the cells were washed twice with PBS and lysed with M-per mammalian extraction buffer (Thermo Fisher Scientific, Rockford, IL.). Following centrifugation at 12,000 g for 10 min, the supernatant of the cell lysate was used for measuring ALP activity using a pNPP Phosphate Assay (Sciencell, Carlsbad, CA.) following manufacturer's instructions. Briefly, a volume of 15 µl of cell lysate was added to 30 µl of assay buffer followed by addition of 5 µl of 10X p-nitorphenyl phosphate (pNPP) solution. The samples were incubated at 37°C for 30-60 minutes. The reaction was stopped by adding of 50 µl of stop solution. Absorbance was mearued using a microtiter plate reader (DTX 880 multimode detector; Beckman Coulter, Fullerton, CA) at 405 nm. The quantity of ALP activity was normalized against the total protein quantity as measured by the BCA protein assay (Thermo Fisher Scientific, Rockford, IL.)

2.6 Statistical Analysis

WST Assay:

A one-way analysis of variance (ANOVA) with Tukey's HSD Post-hoc Pair-wise Comparisons was used for each of the time periods 24, 48, and 72 hours after a single irradiation exposure. These were not repeated measures as each of the time periods was represented by different cell culture wells. The primary outcome measures, comparing control and treatment groups, with treatment groups "normalized" to control groups. Significance for test was predetermined at $P<0.05$.

ALP Assay:

A students T-test was used to performed to determine significant differences in Alkaline Phosphatase (ALP) production. Treatment groups were "normalized" to the control. Significance for test was predetermined at $P<0.05$.

Chapter 3: Results

Cell Proliferation:

The cells irradiated with 6, 12, 18, 24, 30, and 36 seconds were examined at 24, 48 and 72 hours. An additional group was examined at 72 hours for time periods 45, 60, 75, and 90 Seconds. The cell viability at 6 and 12 seconds of exposure was significantly inhibited by 12% and 10%, respectively, after 24 hours when compared to control ($P<0.05$). At 30 seconds of LLLI exposure cell proliferation increased by 18% after 48 hours ($P<0.05$). None of the significant doses mentioned above were significantly different pair-wise.

The 72 hour experimental group demonstrates a dose-responsive curve as explained by Arndt Shulz Law. At this time period 18 seconds of exposure significantly increased proliferation and 60 seconds significantly decreased proliferation by 11% and 27%, respectively. Although the difference between experimental groups at 72 hours was only significant between 6 second and 18 second exposures a bell curve starting low at 6 seconds gradually increasing to 18 seconds and then beginning to decrease again at 24-36 seconds ultimately decreasing proliferation at 60 seconds. It should be noted that at time points 75 and 90 seconds no significant decrease in proliferation was noted which is not expected in relation to the Arndt Shulz Law. Continued decreased proliferation would be expected at these time points. The author speculates a bimodal response may be occurring (Table 3 and Graphs 1-3).

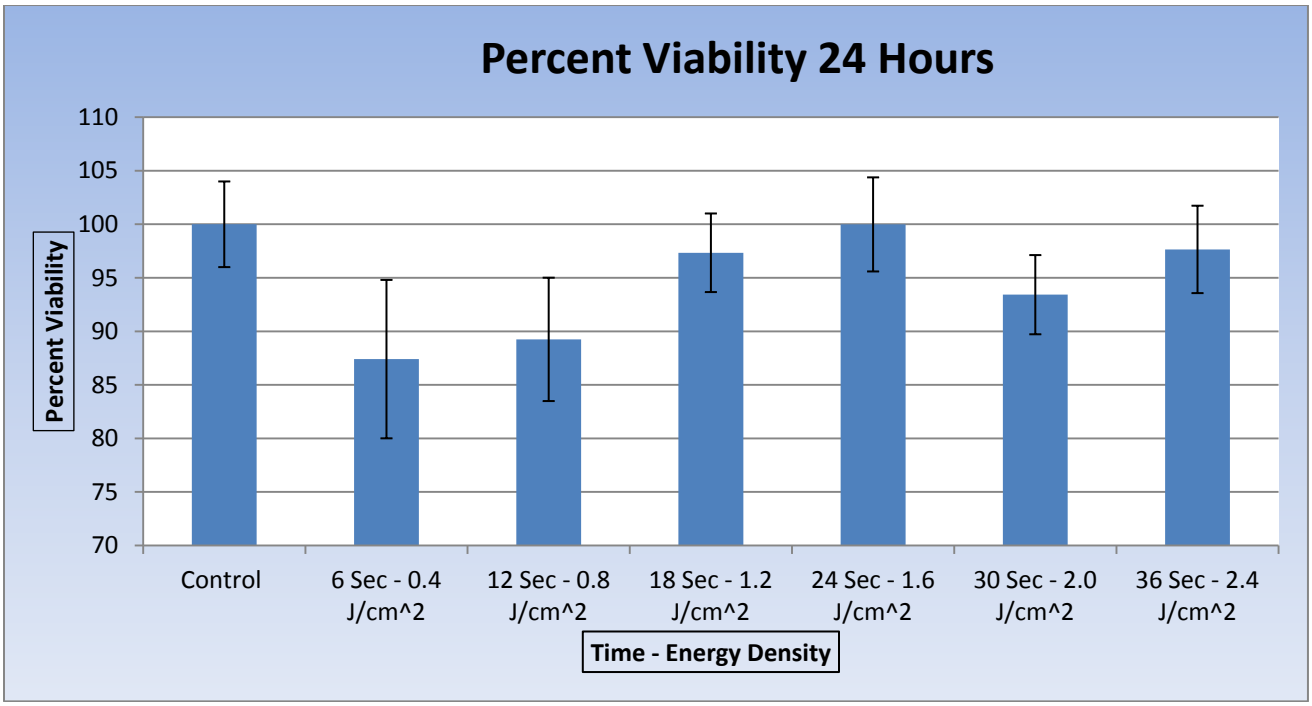
Table 3. Cell Proliferation at 24, 48, and 72 Hours in Percent

	Controls	6 sec	12 sec	18 sec	24 sec	30 sec	36 sec
24 hours	100	88*	90*	98	100	99	98
48 hours	100	106	95	96	99	118*	100
72 hours	100	93	108	111*	106	105	102
J/cm^2	0.00	0.40	0.80	1.20	1.60	2.00	2.40

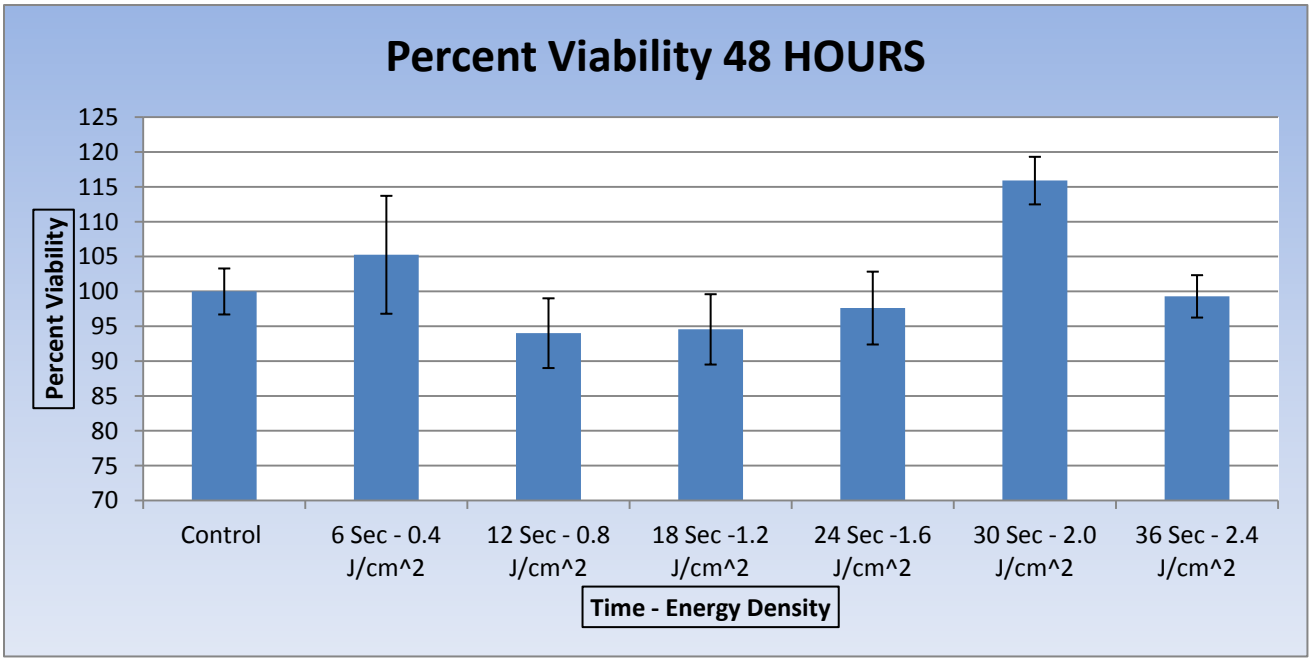
	Controls	45 sec	60 sec	75 sec	90 sec
72 hours Continued	100	90	73*	92	98
J/cm^2	0.00	3.00	4.00	5.00	6.01

The numbers in red with an asterisk show experimental groups that were significantly different than the control. The numbers highlighted in yellow indicate pair wise comparisons demonstrating significant difference between irradiation exposures at the same time.

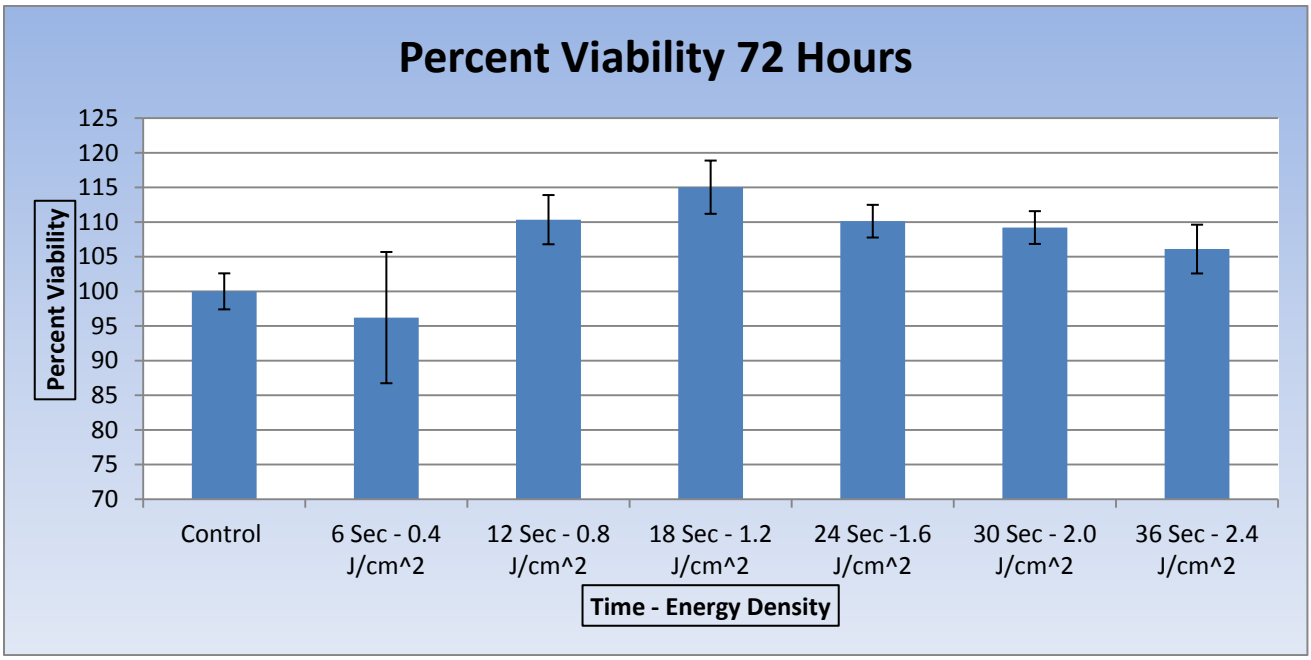
Graph 1. Cell Proliferation 24 Hours after Exposure
Mean±SD n=16



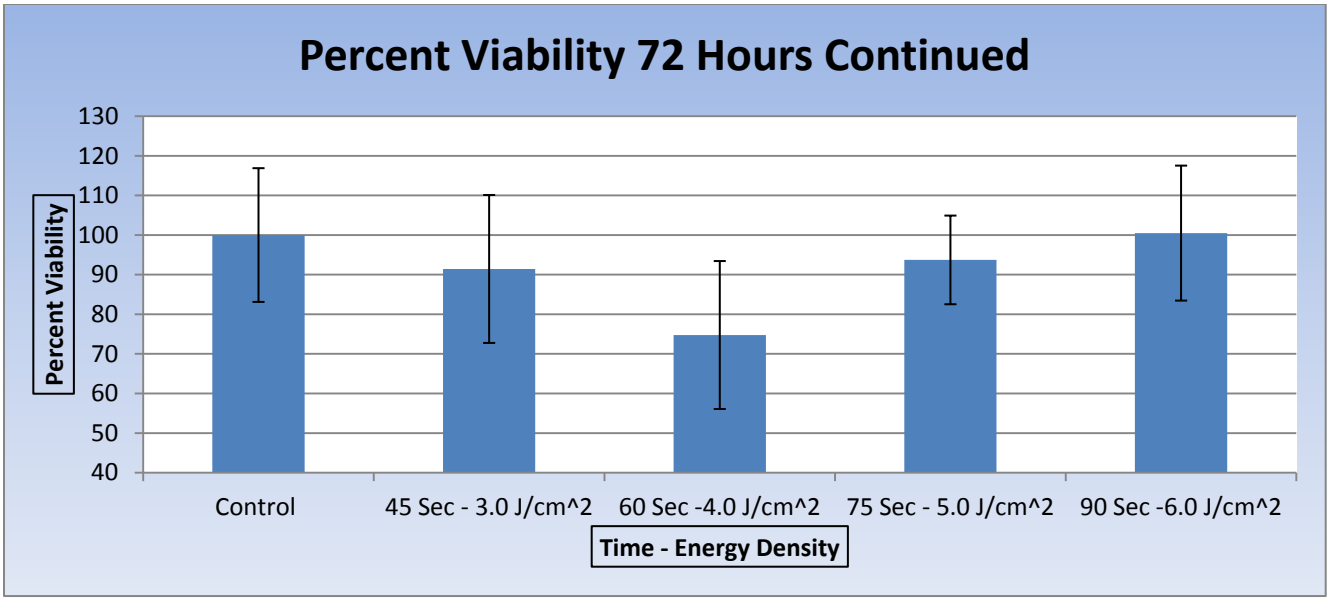
Graph 2. Cell Proliferation 48 Hours after Exposure
Mean±SD n=16



Graph 3. Cell Proliferation 72 Hours after Exposure
Mean±SD n=16



72 Hours Continued



Alkaline Phosphatase Results:

Osteogenic Growth Medium:

Activity of Alkaline phophatase was measured after 10 days of exposure. Our results demonstrated that there was an increase of 59% in ALP activity (P=0.002) when cells were exposed for 48 seconds at 85mW . Similarly, cells exposure for 48 seconds at 269mW showed an increase of a 44% (P=0.001). When these two exposures were compared to each other they were not statistically different but it is interesting to note that the 48 second, 269 mW exposure inhibited ALP activity 15% when compared with the 48 second, 85 mW exposure.

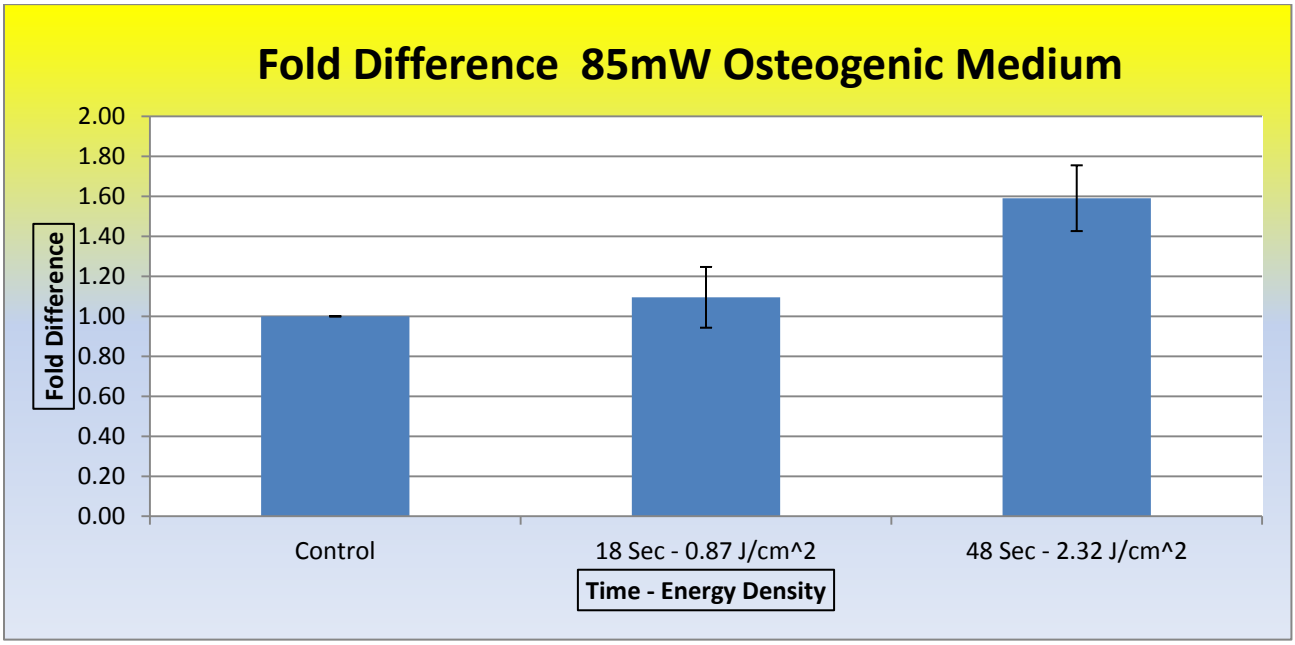
Complete Growth Medium:

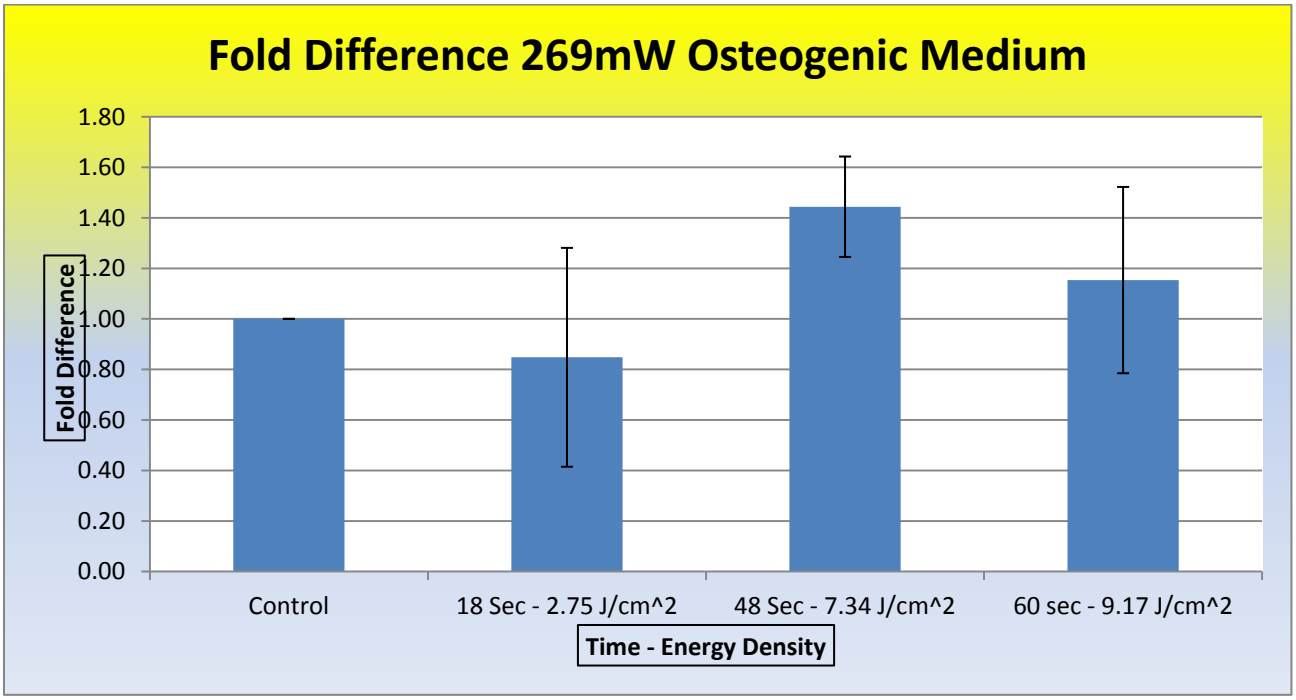
At 10 days ALP activity demonstrated that a 48 and 60 second exposure at 85mW created a 60% and 66% increase, respectively, in ALP activity ($P<0.05$). Similarly the 48 second exposure at 269mW created a 40% increase in ALP activity ($P<0.05$). When these two exposures were compared they were not statistically different but it is interesting to note that the 48 second, 269 mW exposure inhibited ALP activity by 20% when compared with the 48 second, 85 mw exposure.

Both the osteogenic medium and the complete medium studies performed at 269 mW demonstrated a type of dose response curve, as explained by the Arndt Shulz, increasing at 48 seconds and then decreasing at 60 seconds but this difference was not statistically significant.

Graph 4: ALP activity at 10 Days in Osteogenic Medium

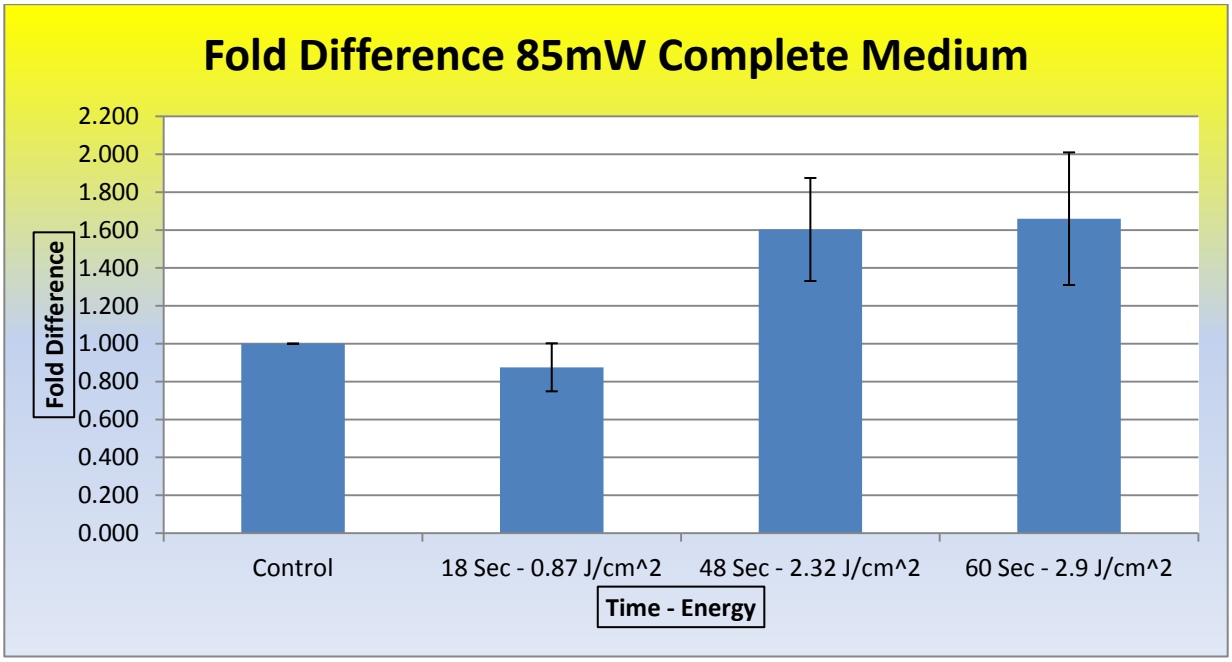
Expressed in Fold Difference

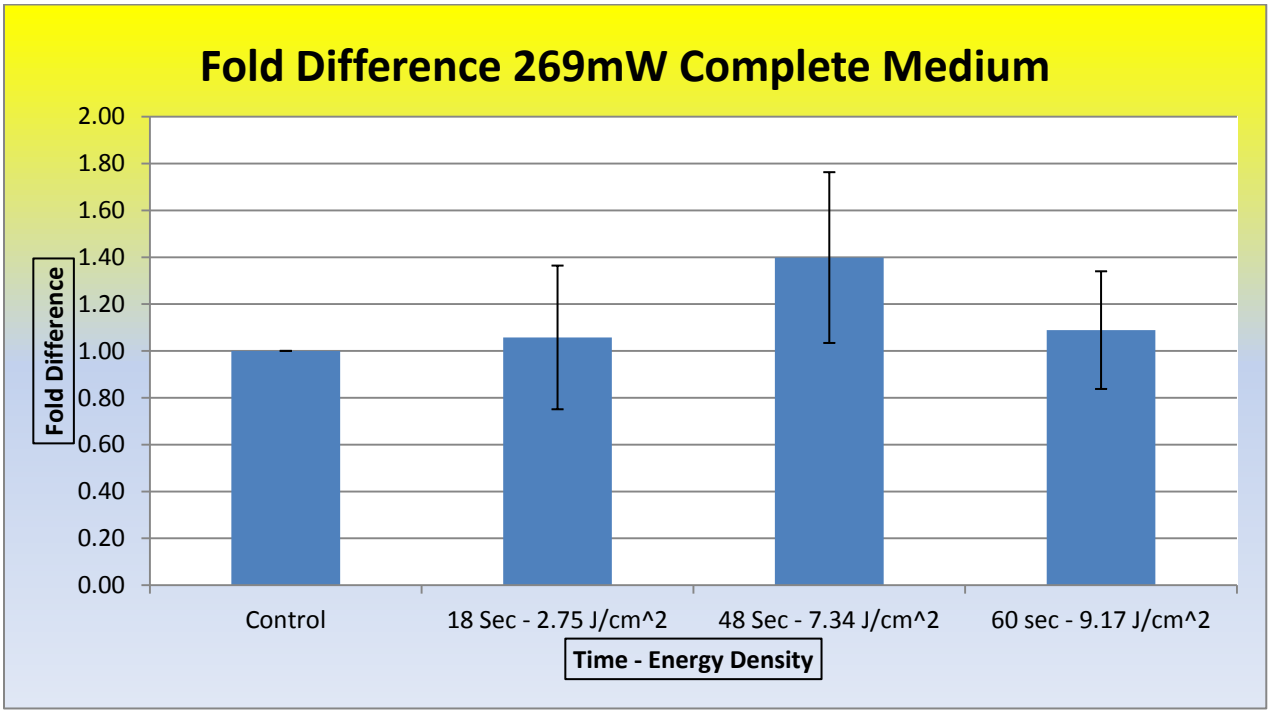




Graph 5: ALP Fold Difference at 10 Days in Complete Medium

Expressed in Fold Difference





Chapter 4: Discussion and Conclusions

Manipulation of the bone and it's mechanisms for remodeling and growth are an important part of orthodontics, and in orthopedics in general. Low Level Laser Irradiation is emerging as an important adjunct to bone therapy. The study was proposed to evaluate the effects of LLLI from the Gallium Aluminum Arsenide Laser (GaAlAs), in the infrared 830 nanometer (nm) range. The project was aimed at establishing the optimum GaALA irradiation parameters such as time of irradiation (dose) and energy densities on human osteoblast cell proliferation and differentiation. The Arndt-Shulz law indicates that weak stimuli excite physiological activity and strong stimuli retard it. The objective is to evaluate applicability of the Arndt-Shulz law, the dose at which the human osteoblast cells proliferate or decrease and the dose at which the differentiation is initiated.

Other *in-vitro* studies have been performed in order to analyze quantitatively and compare LLLI to osteoblast proliferation and alkaline phosphatase production. However, the difficulty found in accurately measuring and explaining the dose of LLLI that the cells receive makes it extremely difficult to extrapolate and compare results amongst published. Appendix A demonstrates the difficulty in extrapolating and comparing the results of many articles. Some of the confounding conditions include, but are not limited to, the complexity of correctly applying a large number of LLLI parameters such as wavelength, power density, pulse structure, total energy, and irradiation timing.[19] Many of the articles reviewed report an energy density and even compare their energy densities with other articles but the majority of the articles fail to report the spot size of the laser or fail to report the distance from the laser to the cell cultures being irradiated. Spot size is the area that the laser light covers as it contacts the cells being irradiated. Without knowing this the optimal dose received by the cells cannot be precisely recorded. The

author found that many of the articles demonstrated in Appendix A assumed the fiber optic width to be the spot size but they did not test for divergence of the laser light. There is also a lack of information given in most articles about the assumptions used in calculating dose.

An example of the difficulty in comparing articles is given here: Laser A reports an energy density of 1.2 J/cm^2, 20 mW, and a 6 second exposure. If laser A is then moved away from the cell culture and the laser diverges from 0.1 to 0.5 cm^2, an energy density of 1.2 J/cm^2, 20 mW, at 6 seconds exposure would be reported. The actual energy density irradiating the cells would be only 0.24 J/cm^2, and it would require a 30 second exposure to actually reach 1.2 J/cm^2. Even if the 30 second exposure was then used at the new spot size the amount of energy that would be emitted to create the same energy density would increase 5 times. Then one has to ask if the cells receive similar treatment as one set received a 6 second exposure and the other a 30 second exposure. This very dilemma was faced as the author contemplated how to deliver a similar dose of LLLI to the 12 well plate at 72 inch for the 10 day ALP testing from the findings in the WST assay.

Alghamdi et al[42] reported that cell proliferation rate at that an energy density of 0.5 to 4.0 J/cm^2, and a visible spectrum ranging from 600 to 700nm of LLLI are optimal for various cell lines."[42] Nevertheless the authors included the beam diameter of the lasers being used but the articles themselves did not reveal adjusting for spot size. Distance from the laser to the cells and calibration of the laser being used was not frequently reported. The similar articles that finally determined various LLLI parameters were to be used for the 24, 48, and 72 hour testing are found in Appendix A. Even amongst these articles great variability is noted concerning the LLLI parameters reported.

One of the limitations in the study was the determination of spot size. We determined the laser spot size 0 .252 cm^2 in contact mode but the area of the of 96 well plate is 0.317 cm^2. As the area of the well is larger than the spot size, it is possible that laser irradiation was not evenly spread to every part of the well of 96 well plate. As noted in materials and methods the cells were irradiated by hand from the bottom of the 96 well plate and it is possible that some movement occurred during irradiation of the cells that may introduce variability into the dose.

The cells were grown in complete or osteogenic medium in 12 well plate for determining activity of alkaline phosphatase. When the cells were exposed to laser irradiation at a distance of 72 inches the authors found variability within the laser beam. The beam showed a type of grid with areas of high and low intensity. The authors were curious to find whether these areas introduced variability within the laser beam itself and so the Sanwa laser detector was positioned in different parts of the rectangular grid and great variability was found. At closer distances (contact mode) it was also noted that the laser's power would fluctuate during an exposure usually only about 5-6 mW over a time period as short as 15 seconds. The authors suspect that these variables have been overlooked by many researchers who have reported findings on the effects of LLLI and may contribute to the confounding reports on the effectiveness of LLLI.

Some of the LLLI parameters used appear to have a proliferative effect on immortalized human osteoblasts at 48 and 72 hours. At 48 hours, it was 30 seconds and 2.0 J/ cm^2 at 72 hours it was 18 seconds at 1.2 J/cm^2. Other authors found similar findings on proliferative effects cells near this range of energy density[42-44]. Their findings do not suggest that these irradiated cells followed a dose response curve as mentioned in the Arndt Shulz Law to a statistically significant level. This result is similar to the sudy of Arisu et al[45] found but our study was performed to a much smaller increment of energy densities. However, the mean cell viability in our study

showed a tendency to follow this dose response curve. Our study may have had too much variability introduced from the weaknesses described earlier to record a statistically significant dose response curve as described by the Arndt Shulz Law.

The ALP findings are intriguing. It appears that the 48 seconds time exposure is just the optimum duration of time to increase the ALP production as it significantly increased production regardless of the 85mw or the 269mw energies. There is a 5 J/cm^2 difference between these two doses but the time appears to be an influencing factor. This was suspected by the author as we see this happen with plants when exposed to too much light they wilt, when exposed to too little they fail to thrive but when given the correct time of light exposure they thrive.

No significant ALP findings were detected in our pilot study for 24, 48, and 72 hours. This was not expected as other studies have reported enhanced ALP activity in this time interval[39, 44, 46]. We attribute our differing findings to the use of a different cell line than the studies cited. We see that the laser makes a difference in proliferation in the 24-72 hours time interval but the cell must not only proliferate to create new bone. The author assumes the cell requires time to perform transcription of DNA and then translation into protein to produce a significant effect upon the ALP.

Conclusions:

Within the limitations of this in-vitro study, it can be concluded that:

1) LLLI has a significant proliferative and inhibitory effect at 24, 48, and 72 hours within the range of doses given.

2) The LLLI significantly affects the ALP activity at 10 days. Alkaline phosphatase activity at 2.4 – 7.3 J/cm^2 with 48 – 60 seconds of exposure, and an incident power ranging from 85-269mw significantly increased. The findings suggest that these irradiated cells obeyed the Arndt Shulz Law governing cellular response to applied energy

3) LLLI should be carefully considered when being used with a patient that has been diagnosed with cancer.

4) It is imperative to establish consistent guidelines for standardization among future in-vitro LLLI studies in order to accurately and consistently compare and evaluate studies.

Appendix A: Literature Review for Dose Requirements

Article	Dose Method	Doses	Effective Doses
Chellini F. Et al [47] Saos-2 Osteoblasts	Nd: Yag Laser PULSED Non contact mode, Laser optic fiber placed 2 mm from irradiation surface. 10 seconds Irradiation Constant 28 degrees C. 400um optic	**Wavelength**:1,064 nm **Pulse duration**: 100 microseconds **Power output**: 1.4 W with 20mJ pulse energy and a variable pulse frequency of 50-70 Hz. **Energy density**: 1.5J/cm^2 **Spot Size: ?** **Beam diameter: ?**	20 mJ and 50Hz 20 mJ and 70Hz Both exerted a strong stimulation of osteoblastic cell proliferation at 24 and 48 hours. MTS assay (mRNA expression of ALP significantly higher at 7 days and then decreased)
Hou et al.[43] BMSC Cells (Used bone marrow derived mesenchymal cells)	InGaAsP diode laser Continuous Wave LLLI Optic fiber placed 89 mm above cells allowing laser beam width of 34mm. Power Density: 6.61mW/cm^2 Irradiation time of 75, 150, 300, and 750 seconds.	**Wavelength**: 635 nm **Power output**: 60 mw, **Total energy**: 4.5, 9, 18, 45 J's **Energy density**: 0, 0.5, 1, 2, or 5 J/cm^2 **Spot size**: ? **Beam diameter**: 34mm	Optimal energy density of **.5 J/cm2** significantly increased BMSC proliferation.
Abramovitch- Gottlib et al. [39] BMSC Cells	Polarized He:NE Laser Continuous Wave Irradiated from a distance of 20 cm for 10 min per day from days 1-28 through a 5 cm hole. Employed intensity: 0.5mW/cm^2	**Wavelength**:632.8 mm **Output Power**: 10 mw **Total Energy**: 6J **Energy Density**: 0.5 J/Cm2 **Spot size**: ? **Beam diameter**: 5cm	ALP significantly enhanced at 48 hours and significantly lower at day 21 and 28. Optimal energy density **0.5J/cm^2 on day 2.**
Kim et al.[46] Mesenchymal Stem	LED Light Continuous Wave MTT assay 96 wells plates and ALP assay. All cells assayed 48 hours after irradiation. Cells exposed 3 cm from the light source.	**Wavelength**:647nm, **Power output**:8.98 to 9.89 mW, **Total energy**: .093, .279 and .836 J, **Energy density**: .01, .03 and .11 J/cm^2, **Spot Size**: ? **Beam Diameter**: 3.2 cm **Time/Energy**: Cells irradiated once for 10 s, 30 s or 90 s at energies .093, .279, and .836 J's.	MTT assay showed no increas in proliferation. ALP assay showed significant increase at 30s at 48 hours. Optimal energy density: **.01, .03, and .11 J/cm^2. The .03 energy density was the most optimal and it was delivered in 30 seconds.**

Renno[48] Mouse osteoblast cells (MC3T3)	GaAlAs diode laser Continuous Wave, Performed on glass Scaffold Irradiating probe was fixed perpendicular above cell culture well at distance of 5mm.	**Wavelength**:830 nm **Power Output**: 30 mW **Energy Density**:10J/Cm2 (at diode) **Spot Size**: 10mm^2 **Beam divergence**:9.5 degree beam divergence, **Time**: 335 s irradiation per spot.	Evaluated cell 7 days after irradiation. Irradiation decreased cell prolifteration 13%. They gave too much for a glass scaffold. Previously they had this dosage worked well in vitro
Stein[49] Human Osteoblasts (Saos 2)	Diode Laser Continuous Wave Evaluated cells at 24, 48, and 72 hrs The laser was adjusted to exactly cover the bottom of one culture well (11.5cm above the bottom of the culture plate.	**Wavelength**: 670 nm **Power output**: 400mW **Energy density**:1J/cm^2 or 2J/cm^2 **Time**: 30 or 60 Seconds **Spot Size**: ?	**1J/cm^2**:Cell viability and alkaline phosphatase slightly enhanced. **2 J/cm^2:** reduced cell viability during the first 48 h and resulted in persistently lower alkaline phosphatase activity mRNA slightly decreased with time in un- treated controls and cells irradiated with 1 J/cm2, but their expression was increased by treatment with 2 J/cm2 after 72 h.
Pires Oliviera [50] Mouse Osteoblasts (Ofcoll II) GaAlAs MTT Assay	GaAlAs Diode Laser Continuous mode Irradiating probe positioned vertically above each well at a distance of 2 cm from the plate. Irradiation time was 36 seconds for each well. Plates positioned in black mask with only area to be irradiated exposed	**Wavelength**: 830 nm **Power output**: 50mW **Energy density**: 3J/cm^2 **Optic Fiber Diameter**: 600um **Spot Size**: ? **Time**: Cells irradiated for 36 seconds at 24 hr intervals with 24, 48 and 72 h incubation times following radiation	MTT assay: Significant increases of living cells of 30% and 50% were observed in comparison to controls after **9.6 minutes (3J/cm^2)** of laser emission (p=0.05) primarily 48 and 72 hrs following the first radiation.
Arisu [45] Human Osteoblasts (Saos 2)	Nd:Yag and HeNE aiming beam Noncontact mode Used MTT to evaluate cells at 7, 14, and 21 days. Lased from a distance of 2mm.	**Wavelength**: 1065 nm **Power output**: Settings varied from 20-120mJ **Pulse repetition rate**: 10-30Hz **Power output**: 0.2-3.6W **Time**: 10 second exposures with 13 different energy settings **Fiber optic diameter**: 320um **Spot Size**: ?	The statistically significant powers were from group 1 with Pulse energy 20MJ at 10 Pulses per second, output power of 0.2 W for 10 seconds of exposure. The other significant group 14 was a HeNe laser continuous wave with 0.1 W power output for 10 seconds.

Stein [44] Human Osteoblasts	HeNe Laser Cells cultured in 24 wells were irradiated using a transparent grid composed of 1.8mm x 1.8mm squares placed at the bottom of the tissue culture plate to ensure precise irradiation over all tissues. Laser irradiation was applied on days 2 and 3 after seeding	**Wavelength**: 632nm **Power Output**: 10mW **Beam diameter**: 1.8mm **Energy Density**: 0.14, 0.43, and 1.43 J/cm^2 **Time**: 1, 3, and 10 seconds **Spot Size**: ?	MTT and ALP assay 24 and 48 hours after second irradiation. MTT: After 3 seconds (0.43 J/cm^2) significant increase of 40% and 38% ALP: After 3 seconds (0.43 J/cm^2) exposure had 2 fold increase in ALP

Appendix B:

WST-1 Descriptive Statistics

24 Hours Descriptive Statistics of Proliferation through Absorbance

Group 24 Hrs	N	Min	Max	Mean	SD	% Viability	P Value
Control (No LLLI)	96	0.232	0.450	0.352	0.040	100.00	N/A
6 Sec of LLLI	16	0.194	0.444	0.307	0.074	87.404	0.029
12 Sec of LLLI	16	0.238	0.491	0.314	0.058	89.250	0.018
18 Sec of LLLI	16	0.292	0.418	0.342	0.037	97.338	0.301
24 Sec of LLLI	16	0.300	0.477	0.351	0.044	99.987	0.963
30 Sec of LLLI	16	0.275	0.387	0.328	0.037	93.425	0.022
36 Sec of LLLI	16	0.281	0.422	0.343	0.041	97.653	0.406

48 Hours Descriptive statistics of Proliferation through Absorbance

Group 48 Hrs	N	Min	Max	Mean	SD	% Viability	P Value
Control (No LLLI)	96	0.236	0.389	0.324	0.033	100.00	N/A
6 Sec of LLLI	16	0.211	0.493	0.341	0.085	105.261	0.421
12 Sec of LLLI	16	0.169	0.371	0.305	0.050	94.016	0.150
18 Sec of LLLI	16	0.158	0.362	0.307	0.050	94.561	0.192
24 Sec of LLLI	16	0.143	0.376	0.317	0.052	97.619	0.585
30 Sec of LLLI	16	0.308	0.425	0.376	0.034	115.907	0.000
36 Sec of LLLI	16	0.270	0.393	0.322	0.030	99.297	0.810

72 Hours Descriptive statistics of Proliferation through Absorbance

Group 72 Hrs	N	Min	Max	Mean	SD	% Viability	P Value
Control (No LLLI)	72	0.201	0.420	0.309	0.026	100.000	N/A
6 Sec of LLLI	20	0.140	0.433	0.297	0.095	96.208	0.587
12 Sec of LLLI	20	0.276	0.405	0.341	0.036	110.347	0.001
18 Sec of LLLI	20	0.295	0.424	0.355	0.038	115.032	0.000
24 Sec of LLLI	20	0.306	0.397	0.340	0.024	110.127	0.000
30 Sec of LLLI	20	0.287	0.380	0.337	0.024	109.208	0.000
36 Sec of LLLI	20	0.255	0.368	0.327	0.035	106.104	0.027

72 Hours Descriptive statistics of Proliferation through Absorbance (Continued)

Group 72 HRS	N	Min	Max	Mean Absorbance	SD	Percent Viability	P Value
Control (No LLLI)	80	0.301	0.930	0.624	0.169	100.000	N/A
45 Sec of LLLI	16	0.283	0.860	0.570	0.187	91.432	0.270
60 Sec of LLLI	16	0.262	0.887	0.466	0.187	74.758	0.003
75 Sec of LLLI	16	0.372	0.722	0.585	0.112	93.722	0.182
90 Sec of LLLI	16	0.306	0.888	0.627	0.170	100.488	0.944

Appendix C:

10 Day ALP Descriptive Statistics

	Osteogenic Medium					
		85 mW		269 mW		
	Control	18 Sec – 0.87 J/cm^2	48 Sec – 2.32 J/cm^2	18 Sec – 2.75 J/cm^2	48 Sec – 7.34 J/cm^2	60 Sec – 9.17 J/cm^2
		8.00	8.00	8.00	8.00	8.00
Mean	100.0	109.50	159.08	84.80	144.39	115.36
SD		15.19	16.45	43.31	19.88	36.87
SEM		5.37	5.82	15.31	7.03	13.04
P Value		**P=0.206**	**P=0.002**	**P= 0.262**	**P=0.001**	**P= 0.171**

	Complete Medium					
	85 mW			269 mW		
	18 Sec- 0.9 J/cm^2	48 Sec- 2.3 J/cm^2	60 Sec- 2.9 J/cm^2	18 Sec- 2.8 J/cm^2	48 Sec- 7.34 J/cm^2	60 Sec- 9.2 J/cm^2
	8.00	8.00	8.00	8.00	8.00	8.00
Mean	87.54	160.30	166.44	105.76	139.85	108.87
SD	12.67	27.19	34.75	30.67	36.47	25.12
SEM	4.48	9.61		10.84	12.89	8.88
P Value	**P=0.109**	**P=0.049**	**P=0.005**	**P=0.240**	**p=0.046**	**p=0.464**

Appendix D: Raw Data

WST ASSAY 24 HOURS AFTER SINGLE DOSE OF LLLI

N	6 Sec	12 Sec	18 Sec	24 Sec	30 Sec	36 Sec	Control	Control	Control	Control	Control	Control
1	0.217	0.308	0.367	0.388	0.367	0.409	0.365	0.413	0.381	0.257	0.352	0.336
2	0.295	0.343	0.375	0.376	0.387	0.422	0.424	0.415	0.397	0.270	0.386	0.388
3	0.325	0.297	0.391	0.477	0.370	0.383	0.374	0.380	0.393	0.392	0.401	0.369
4	0.419	0.356	0.376	0.409	0.358	0.333	0.363	0.372	0.399	0.304	0.417	0.367
5	0.444	0.491	0.418	0.349	0.362	0.281	0.305	0.371	0.370	0.279	0.389	0.350
6	0.430	0.243	0.360	0.300	0.336	0.345	0.343	0.372	0.395	0.308	0.404	0.335
7	0.323	0.238	0.359	0.326	0.350	0.349	0.379	0.360	0.365	0.318	0.353	0.296
8	0.295	0.283	0.318	0.338	0.337	0.361	0.354	0.280	0.368	0.232	0.286	0.418
9	0.309	0.323	0.329	0.346	0.332	0.385	0.352	0.339	0.357	0.299	0.285	0.329
10	0.306	0.317	0.335	0.335	0.339	0.353	0.350	0.354	0.344	0.340	0.334	0.357
11	0.325	0.302	0.302	0.346	0.300	0.325	0.327	0.336	0.336	0.394	0.356	0.364
12	0.294	0.314	0.304	0.347	0.287	0.325	0.306	0.389	0.313	0.360	0.450	0.377
13	0.209	0.322	0.292	0.339	0.294	0.303	0.301	0.309	0.316	0.375	0.449	0.339
14	0.194	0.309	0.324	0.305	0.286	0.289	0.323	0.297	0.329	0.365	0.358	0.344
15	0.276	0.318	0.304	0.320	0.275	0.318	0.327	0.287	0.311	0.380	0.359	0.398
16	0.255	0.256	0.321	0.323	0.276	0.313	0.310	0.302	0.310	0.409	0.360	0.395

WST ASSAY 48 HOURS AFTER SINGLE DOSE OF LLLI

N	6 Sec	12 Sec	18 Sec	24 Sec	30 Sec	36 Sec	Control	Control	Control	Control	Control	Control
1	0.347	0.169	0.362	0.341	0.403	0.326	0.298	0.329	0.276	0.323	0.303	0.303
2	0.493	0.265	0.342	0.342	0.408	0.318	0.314	0.254	0.322	0.309	0.325	0.316
3	0.384	0.313	0.323	0.321	0.425	0.315	0.282	0.287	0.349	0.291	0.316	0.277
4	0.386	0.278	0.306	0.376	0.425	0.302	0.327	0.312	0.330	0.325	0.275	0.252
5	0.389	0.323	0.300	0.338	0.408	0.291	0.296	0.303	0.353	0.301	0.344	0.270
6	0.353	0.255	0.158	0.314	0.394	0.302	0.306	0.236	0.334	0.339	0.283	0.250
7	0.420	0.312	0.317	0.356	0.388	0.321	0.322	0.329	0.328	0.324	0.272	0.279
8	0.474	0.293	0.296	0.350	0.360	0.270	0.331	0.307	0.247	0.299	0.325	0.311
9	0.223	0.365	0.319	0.143	0.308	0.339	0.335	0.338	0.256	0.362	0.357	0.311
10	0.294	0.337	0.289	0.344	0.372	0.305	0.354	0.335	0.361	0.351	0.307	0.346
11	0.347	0.277	0.233	0.299	0.361	0.355	0.369	0.379	0.329	0.348	0.389	0.355
12	0.354	0.316	0.311	0.316	0.361	0.365	0.342	0.352	0.344	0.340	0.379	0.290
13	0.298	0.371	0.339	0.305	0.360	0.393	0.325	0.371	0.375	0.384	0.373	0.344
14	0.269	0.337	0.344	0.302	0.372	0.338	0.309	0.348	0.350	0.343	0.376	0.376
15	0.223	0.357	0.361	0.338	0.352	0.316	0.331	0.349	0.346	0.360	0.369	0.310
16	0.211	0.312	0.307	0.283	0.319	0.301	0.309	0.344	0.313	0.346	0.345	0.333

WST ASSAY 72 HOURS AFTER SINGLE DOSE OF LLLI

N	6 Sec	12 Sec	18 Sec	24 Sec	30 Sec	36 Sec	Control	Control	Control	Control	Control	Control
1	0.140	0.309	0.296	0.318	0.350	0.265	0.271	0.350	0.312	0.293	0.235	0.277
2	0.141	0.312	0.334	0.306	0.313	0.331	0.332	0.309	0.370	0.337	0.298	0.256
3	0.198	0.355	0.295	0.314	0.348	0.353	0.348	0.323	0.380	0.334	0.262	0.236
4	0.280	0.305	0.332	0.327	0.335	0.347	0.360	0.326	0.374	0.320	0.260	0.244
5	0.266	0.324	0.302	0.310	0.317	0.368	0.323	0.342	0.370	0.343	0.304	0.236
6	0.207	0.320	0.354	0.331	0.328	0.367	0.318	0.342	0.356	0.307	0.281	0.218
7	0.192	0.346	0.312	0.315	0.323	0.314	0.336	0.299	0.345	0.316	0.261	0.201
8	0.214	0.343	0.343	0.357	0.356	0.337	0.343	0.347	0.318	0.351	0.275	0.226
9	0.329	0.328	0.349	0.327	0.326	0.349	0.341	0.352				
10	0.381	0.324	0.351	0.345	0.314	0.293	0.272	0.319				
11	0.418	0.405	0.399	0.363	0.343	0.359	0.396	0.420				
12	0.402	0.361	0.405	0.355	0.369	0.341	0.347	0.365				
13	0.295	0.397	0.363	0.397	0.356	0.341	0.301	0.359				
14	0.309	0.373	0.348	0.370	0.372	0.350	0.338	0.332				
15	0.421	0.399	0.424	0.362	0.346	0.351	0.322	0.346				
16	0.415	0.321	0.336	0.334	0.314	0.302	0.317	0.354				
17	0.433	0.365	0.373	0.361	0.347	0.279	0.324	0.366				
18	0.261	0.296	0.393	0.341	0.316	0.288	0.284	0.356				
19	0.319	0.276	0.409	0.329	0.380	0.362	0.291	0.316				
20	0.317	0.353	0.383	0.334	0.287	0.255	0.237	0.379				

WST ASSAY 72 HOURS AFTER SINGLE DOSE OF LLLI (More Time Points)

N	45 Sec	60 Sec	75 Sec	90 Sec	Control	Control	Control	Control	Control
1	0.757	0.887	0.644	0.702	0.754	0.558	0.781	0.785	0.573
2	0.718	0.550	0.718	0.707	0.702	0.536	0.800	0.794	0.841
3	0.727	0.429	0.630	0.888	0.655	0.553	0.727	0.812	0.800
4	0.314	0.419	0.610	0.640	0.694	0.549	0.582	0.667	0.646
5	0.519	0.347	0.519	0.571	0.605	0.724	0.539	0.582	0.342
6	0.475	0.284	0.509	0.679	0.515	0.536	0.632	0.650	0.311
7	0.283	0.362	0.572	0.668	0.611	0.459	0.487	0.429	0.656
8	0.349	0.436	0.374	0.545	0.573	0.323	0.301	0.317	0.414
9	0.824	0.746	0.663	0.719	0.536	0.822	0.729	0.825	0.729
10	0.860	0.536	0.546	0.745	0.639	0.771	0.785	0.869	0.847
11	0.709	0.648	0.696	0.788	0.745	0.729	0.770	0.840	0.893
12	0.666	0.359	0.602	0.773	0.476	0.779	0.713	0.777	0.930
13	0.356	0.340	0.699	0.619	0.590	0.713	0.485	0.647	0.877
14	0.571	0.330	0.722	0.370	0.693	0.717	0.385	0.339	0.750
15	0.497	0.525	0.372	0.306	0.349	0.701	0.335	0.741	0.345
16	0.501	0.262	0.477	0.307	0.422	0.424	0.666	0.388	0.318

ALP 10 DAYS AFTER SINGLE DOSE OF LLLI

ALP assay Results													
Osteogenic Medium							Complete Medium						
85 mW			269 mW				85 mW			269 mW			
Control	18 Sec	48 Sec	Control	18 sec	48 Sec	60 Sec	Control	18 Sec	48 Sec	Control	18 sec	48 Sec	60 Sec
0.113	0.084	0.072	0.113	0.063	0.059	0.073	0.067	0.058	0.082	0.067	0.051	0.063	0.062
0.119	0.090	0.071	0.119	0.072	0.083	0.083	0.060	0.072	0.078	0.060	0.053	0.087	0.072
0.065	0.076	0.065	0.065	0.066	0.045	0.046	0.079	0.069	0.086	0.079	0.068	0.080	0.069
0.058	0.077	0.067	0.058	0.055	0.040	0.042	0.092	0.080	0.088	0.092	0.054	0.104	0.078
0.082	0.061	0.041	0.082	0.054	0.056	0.045	0.082	0.061	0.041	0.082	0.054	0.056	0.045
0.079	0.055	0.030	0.079	0.046	0.054	0.040	0.079	0.055	0.030	0.079	0.046	0.054	0.040
0.045	0.053	0.058	0.045	0.035	0.032	0.034	0.045	0.053	0.058	0.045	0.035	0.032	0.034
0.047	0.042	0.046	0.047	0.042	0.029	0.031	0.047	0.042	0.046	0.047	0.042	0.029	0.031

Protein Content in in µg per 15 µL													
8.97	5.29	3.49	8.97	10.22	3.67	3.88	10.98	13.61	8.73	10.98	10.50	8.52	7.78
8.97	5.29	3.49	8.97	10.22	3.67	3.88	10.98	13.61	8.73	10.98	10.50	8.52	7.78
10.59	12.83	6.42	10.59	8.22	5.52	8.15	14.93	13.69	7.56	14.93	8.59	8.20	11.11
10.59	12.83	6.42	10.59	8.22	5.52	8.15	14.93	13.69	7.56	14.93	8.59	8.20	11.11
8.97	5.29	3.49	8.97	10.22	3.67	3.88	10.98	13.61	8.73	10.98	10.50	8.52	7.78
8.97	5.29	3.49	8.97	10.22	3.67	3.88	10.98	13.61	8.73	10.98	10.50	8.52	7.78
10.59	12.83	6.42	10.59	8.22	5.52	8.15	14.93	13.69	7.56	14.93	8.59	8.20	11.11
10.59	12.83	6.42	10.59	8.22	5.52	8.15	14.93	13.69	7.56	14.93	8.59	8.20	11.11

Normalization													
0.013	0.016	0.021	0.013	0.006	0.016	0.019	0.006	0.004	0.009	0.006	0.005	0.007	0.008
0.013	0.017	0.020	0.013	0.007	0.023	0.021	0.005	0.005	0.009	0.005	0.005	0.010	0.009
0.006	0.006	0.010	0.006	0.008	0.008	0.006	0.005	0.005	0.011	0.005	0.008	0.010	0.006
0.005	0.006	0.010	0.005	0.007	0.007	0.005	0.006	0.006	0.012	0.006	0.006	0.013	0.007
0.009	0.012	0.012	0.009	0.005	0.015	0.012	0.007	0.004	0.005	0.007	0.005	0.007	0.006
0.009	0.010	0.009	0.009	0.004	0.015	0.010	0.007	0.004	0.003	0.007	0.004	0.006	0.005
0.004	0.004	0.009	0.004	0.004	0.006	0.004	0.003	0.004	0.008	0.003	0.004	0.004	0.003
0.004	0.003	0.007	0.004	0.005	0.005	0.004	0.003	0.003	0.006	0.003	0.005	0.004	0.003

Fold difference													
100	126	165		49	129	150		71	154		81	122	131
100	128	152		53	171	161		97	165		93	188	169
100	96	164		130	133	91		96	216		151	185	118
100	110	191		122	133	93		95	189		101	206	114
100	126	128		58	167	127		60	63		69	88	77
100	118	97		51	167	117		56	48		61	88	71
100	97	213		100	137	98		128	255		135	129	102
100	74	162		115	118	86		97	193		155	112	89

BIBLIOGRAPHY

1. Spadaro JA. Mechanical and electrical interactions in bone remodeling. Bioelectromagnetics 1997;18(3):193-202.
2. Hadjiargyrou M, McLeod K, Ryaby JP, Rubin C. Enhancement of fracture healing by low intensity ultrasound. Clin Orthop Relat Res 1998(355 Suppl):S216-29.
3. Kobayashi Y, Takagi H, Sakai H, Hashimoto F, Mataki S, Kobayashi K, et al. Effects of local administration of osteocalcin on experimental tooth movement. Angle Orthod 1998;68(3):259-66.
4. Seifi M, Eslami B, Saffar AS. The effect of prostaglandin E2 and calcium gluconate on orthodontic tooth movement and root resorption in rats. Eur J Orthod 2003;25(2):199-204.
5. Madan MS, Liu ZJ, Gu GM, King GJ. Effects of human relaxin on orthodontic tooth movement and periodontal ligaments in rats. Am J Orthod Dentofacial Orthop 2007;131(1):8 e1-10.
6. Kale S, Kocadereli I, Atilla P, Asan E. Comparison of the effects of 1,25 dihydroxycholecalciferol and prostaglandin E2 on orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2004;125(5):607-14.
7. Cruz DR, Kohara EK, Ribeiro MS, Wetter NU. Effects of low-intensity laser therapy on the orthodontic movement velocity of human teeth: a preliminary study. Lasers Surg Med 2004;35(2):117-20.
8. Doshi-Mehta G, Bhad-Patil WA. Efficacy of low-intensity laser therapy in reducing treatment time and orthodontic pain: A clinical investigation. Am J Orthod Dentofacial Orthop 2012;141(3):289-97.
9. Kawasaki K, Shimizu N. Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg Med 2000;26(3):282-91.
10. Renno AC, McDonnell PA, Parizotto NA, Laakso EL. The effects of laser irradiation on osteoblast and osteosarcoma cell proliferation and differentiation in vitro. Photomed Laser Surg 2007;25(4):275-80.
11. Graber TM. Orthodontics Current Principles and Techniques. In: Vanarsdall RL, editor. 11830 Westline Industrial Drive, St. Louis, Missouri 63146: Elsevier Mosby; 2005. p. 149-68.
12. Proffit WR. Contemporary Orthodontics In: HW F, editor. Fourth Edition ed. 11830 Westline Industrial Drive, St. Louis, Missouri 63146: Mosby, Inc., an affiliate of Elsevier Inc.; 2007. p. 338.
13. Borke J HX, Winger J, Orellana M, Do N,. FEA/Histology technique for assessing tissue response to mechanical loading in orthodontic tooth movement. Harvard Society for the Advancement of Orthodontics, Boston, Massachussetts, USA, Edited by Davidovitch Z, Mah J, and Suthanarak S. 2006;Biological Mechanisms of Tooth Eruption, Resoprtion, and Movement:261-65.
14. Phan TC, Xu J, Zheng MH. Interaction between osteoblast and osteoclast: impact in bone disease. Histol Histopathol 2004;19(4):1325-44.

15. Dobnig H, Turner RT. Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 1995;136(8):3632-8.
16. Owens JM, Gallagher AC, Chambers TJ. Bone cells required for osteoclastic resorption but not for osteoclastic differentiation. Biochem Biophys Res Commun 1996;222(2):225-9.
17. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. J Cell Sci 2000;113 (Pt 3):377-81.
18. Pick LJMRM, editor. Lasers in Dentistry; 1995.
19. Huang YY, Chen AC, Carroll JD, Hamblin MR. Biphasic dose response in low level light therapy. Dose Response 2009;7(4):358-83.
20. Karu T, editor. Ten Lectures on Basic Science of Laser Phototherapy. Grangersberg; 2007.
21. Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol B 1999;49(1):1-17.
22. Mester E, Szende B, Gartner P. [The effect of laser beams on the growth of hair in mice]. Radiobiol Radiother (Berl) 1968;9(5):621-6.
23. dos Santos Lde F, Carvalho Ade A, Leao JC, Cruz Perez DE, Castro JF. Effect of low-level laser therapy in the treatment of burning mouth syndrome: a case series. Photomed Laser Surg 2011;29(12):793-6.
24. Igic M, Kesic L, Lekovic V, Apostolovic M, Mihailovic D, Kostadinovic L, et al. Chronic gingivitis: the prevalence of periodontopathogens and therapy efficiency. Eur J Clin Microbiol Infect Dis 2012.
25. da Silva AP, Petri AD, Crippa GE, Stuani AS, Rosa AL, Stuani MB. Effect of low-level laser therapy after rapid maxillary expansion on proliferation and differentiation of osteoblastic cells. Lasers Med Sci 2011.
26. Jovanovic G, Uric N, Krunic N, Tijanic M, Stojanovic S. [Assessment of the effectiveness of low level laser in the treatment of alveolar osteitis]. Vojnosanit Pregl 2011;68(6):506-10.
27. Park JJ, Kang KL. Effect of 980-nm GaAlAs diode laser irradiation on healing of extraction sockets in streptozotocin-induced diabetic rats: a pilot study. Lasers Med Sci 2012;27(1):223-30.
28. Tanboga I, Eren F, Altinok B, Peker S, Ertugral F. The effect of low level laser therapy on pain during dental tooth-cavity preparation in children. Eur Arch Paediatr Dent 2011;12(2):93-5.
29. Sousa MV, Scanavini MA, Sannomiya EK, Velasco LG, Angelieri F. Influence of low-level laser on the speed of orthodontic movement. Photomed Laser Surg 2011;29(3):191-6.
30. Sgolastra F, Petrucci A, Gatto R, Monaco A. Effectiveness of laser in dentinal hypersensitivity treatment: a systematic review. J Endod 2011;37(3):297-303.
31. Oz S, Gokcen-Rohlig B, Saruhanoglu A, Tuncer EB. Management of myofascial pain: low-level laser therapy versus occlusal splints. J Craniofac Surg 2010;21(6):1722-8.
32. Limpanichkul W, Godfrey K, Srisuk N, Rattanayatikul C. Effects of low-level laser therapy on the rate of orthodontic tooth movement. Orthod Craniofac Res 2006;9(1):38-43.

33. Yoshida T, Yamaguchi M, Utsunomiya T, Kato M, Arai Y, Kaneda T, et al. Low-energy laser irradiation accelerates the velocity of tooth movement via stimulation of the alveolar bone remodeling. Orthod Craniofac Res 2009;12(4):289-98.
34. Abi-Ramia LB, Stuani AS, Stuani MB, Mendes Ade M. Effects of low-level laser therapy and orthodontic tooth movement on dental pulps in rats. Angle Orthod 2010;80(1):116-22.
35. Seifi M, Shafeei HA, Daneshdoost S, Mir M. Effects of two types of low-level laser wave lengths (850 and 630 nm) on the orthodontic tooth movements in rabbits. Lasers Med Sci 2007;22(4):261-4.
36. Tuner J. The New Laser Therapy Handbook. In: Hode L, editor. A guide for research scientists, doctors, dentists, veterinarians and other interested parties within the medical field. Spjutvagen 11, 772 32 Grangesberg, Sweden: Prima Books AB; 2010. p. 514.
37. Da Cunha SS, Sarmento V, Ramalho LM, De Almeida D, Veeck EB, Da Costa NP, et al. Effect of laser therapy on bone tissue submitted to radiotherapy: experimental study in rats. Photomed Laser Surg 2007;25(3):197-204.
38. Zimin AA, Zhevago NA, Buiniakova AI, Samoilova KA. [Application of low-power visible and near infrared radiation in clinical oncology]. Vopr Kurortol Fizioter Lech Fiz Kult 2009(6):49-52.
39. Abramovitch-Gottlib L, Gross T, Naveh D, Geresh S, Rosenwaks S, Bar I, et al. Low level laser irradiation stimulates osteogenic phenotype of mesenchymal stem cells seeded on a three-dimensional biomatrix. Lasers Med Sci 2005;20(3-4):138-46.
40. Shupnik MA, Antoniades HN, Tashjian AH, Jr. Platelet-derived growth factor increases prostaglandin production and decreases epidermal growth factor receptors in human osteosarcoma cells. Life Sci 1982;30(4):347-53.
41. Shupnik MA, Tashjian AH, Jr. Epidermal growth factor and phorbol ester actions on human osteosarcoma cells. Characterization of response and nonresponsive cell lines. J Biol Chem 1982;257(20):12161-4.
42. Alghamdi KM, Kumar A, Moussa NA. Low-level laser therapy: a useful technique for enhancing the proliferation of various cultured cells. Lasers Med Sci 2012;27(1):237-49.
43. Hou JF, Zhang H, Yuan X, Li J, Wei YJ, Hu SS. In vitro effects of low-level laser irradiation for bone marrow mesenchymal stem cells: proliferation, growth factors secretion and myogenic differentiation. Lasers Surg Med 2008;40(10):726-33.
44. Stein A, Benayahu D, Maltz L, Oron U. Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. Photomed Laser Surg 2005;23(2):161-6.
45. Arisu HD, Turkoz E, Bala O. Effects of Nd:Yag laser irradiation on osteoblast cell cultures. Lasers Med Sci 2006;21(3):175-80.
46. Kim HK, Kim JH, Abbas AA, Kim DO, Park SJ, Chung JY, et al. Red light of 647 nm enhances osteogenic differentiation in mesenchymal stem cells. Lasers Med Sci 2009;24(2):214-22.
47. Chellini F, Sassoli C, Nosi D, Deledda C, Tonelli P, Zecchi-Orlandini S, et al. Low pulse energy Nd:YAG laser irradiation exerts a biostimulative effect on different cells of the oral microenvironment: "an in vitro study". Lasers Surg Med 2010;42(6):527-39.
48. Renno AC, McDonnell PA, Crovace MC, Zanotto ED, Laakso L. Effect of 830 nm laser phototherapy on osteoblasts grown in vitro on Biosilicate scaffolds. Photomed Laser Surg 2010;28(1):131-3.

49. Stein E, Koehn J, Sutter W, Wendtlandt G, Wanschitz F, Thurnher D, et al. Initial effects of low-level laser therapy on growth and differentiation of human osteoblast-like cells. Wien Klin Wochenschr 2008;120(3-4):112-7.
50. Pires Oliveira DA, de Oliveira RF, Zangaro RA, Soares CP. Evaluation of low-level laser therapy of osteoblastic cells. Photomed Laser Surg 2008;26(4):401-4.