Musbahu Muhammad SANI

EFFECTS OF LOW LEVEL LASER THERAPY ON WOUND HEALING RATE IN RATS USING PULSED LASER OPERATING MODE

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By

Musbahu Muhammad SANI

JANUARY 2014

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FATİH ÜNİVESİTESİ
BİYOMEDİKAL MÜHENDİSLİK ENSTİTÜSÜ

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YÜKSEK LİSANS TEZİ
BİYOMEDİKAL MÜHENDİSLİĞİ PROGRAMI

DANIŞMAN
YRD. DOÇ. DR. HAŞİM ÖZGÜR TABAKOĞLU

ISTANBUL, OCAK / 2014

T.C.

FATIH UNIVERSITY

INSTITUTE OF BIOMEDICAL ENGINEERING

MUSBAHU MUHAMMAD SANI, an **MSc** student of Fatih University **Institute of Biomedical Engineering** student ID **520112026**, successfully defended the **thesis** entitled **EFFECTS OF LOW LEVEL LASER THERAPY ON WOUND HEALING RATE IN RATS USING PULSED LASER OPERATING MODE**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor: **Asst. Prof. Haşim Özgür TABAKOĞL**.....

Fatih University

Jury Members: **Prof. Dr. Ata AKIN** **……..**.....

Bilgi University

Asst. Prof. Şükrü OKKESİM…..…….....

Fatih University

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Date of Defense: **20 January 2014**

To my family for all the prayers, support and assistance.

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In the name of Allah, the Most Beneficent, the Most Merciful. All praises and Thanks be to Him Whom we worship and unto Whom we seek assistance, and by Whose will I have yet successfully completed this master of science (MSc) research thesis.

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January 2014 Musbahu Muhammad SANI

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LIST OF SYMBOLS

α	Alpha
β	Beta
λ	Lambda (Wavelength)

ABBREVATIONS

AlGaInP	Aluminium Gallium Indium Phosphide
ATP	Adenosine Triphosphate
CCD	Charge-coupled device
Cox	Cytochrome c oxidase
CW	Continuous Wave
ECM	Extra Cellular Membrane
EMT	Epithelial Mesenchymal Transition
EGF	Epidermal Growth Factor
FGF	Fibroblast growth factor
FDA	Food and Drug Administration
GaAlAs	Gallium Aluminum Arsenide Laser
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GF	Growth Factors
HeNe	Helium Neon Laser
HE	Hematoxylin Eosin
IFN	Interferons Alpha and Beta
IL	Interleukins
pHi	Intracellular pH
PVA	Polyvinyl Alcohol
LED	Light Emitting Diode
LLLI	Low Level Laser Irradiation
LLLT	Low Level Laser Therapy
MMPs	Matrix Metalloproteinases
NIR	Near Infrared
NADPH	Nicotinamide Adenine dinucleotide Phosphate Reduced
NADH	Nicotinamide Adenine dinucleotide Reduced
NASA	National Aeronautics and Space Administration
PDGF	Platelet-derived growth factor
RNA	Ribonucleic Acid
TGF	Transforming growth factors
TNF	Tumor necrosis growth factor
VEGF	Vascular Endothelial Growth Factor

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SUMMARY

Effects Of Low Level Laser Therapy On Wound Healing Rate In Rats Using Pulsed Laser Operating Mode

Musbahu Muhammad SANI

Biomedical Engineering Programme

MSc Thesis

Advisor: Asst. Prof. Haşim Özgur TABAKOĞLU

Low-level laser therapy is an important method for the treatment of healing processes. The effects of pulsed LLLI on punch wound healing rate between groups irradiated immediately after wounding, and those irradiated later post wounding were histologically and morphometrically compared. 12 wistar rats were divided into four groups receiving laser irradiation ($5J/cm^2$, $0.02W/cm^2$ from 808nm) immediately after wounding (inflammatory group), 24 hrs post wounding (proliferative group), 72 hrs post wounding (remodeling group) and no irradiation (Control group). Animals were sacrificed and tissue samples obtained subjected to histological analysis on 3rd, 7th and 14th days post wounding to monitor our aim. Mean WHR for all treatment groups were found to differ significantly from the control group with lower mean value ($p>0.05$) from the control than tests. No statistical significant difference ($p>0.05$) was found between proliferative and inflammatory groups with the former having higher mean value. Upon comparing proliferative and remodeling groups, significant difference was found with higher mean value from proliferative group. However there was no significant difference found between inflammatory and remodeling groups with the former having slightly higher mean value. From the histological and morphometrical results obtained, we concluded that LLLT has best effect when first applied about 24hrs post wounding (late inflammatory, early proliferative stage) as evidenced by increase in granulation tissue, fibroblasts and collagen deposition leading to faster wound contracture and thus healings.

Keywords**:** LLLT, Inflammatory, Proliferative, Remodeling, Wound healing, Wound contraction, GaAlAs.

ÖZET

Sicanlar Üzerindedarbeli Moduyeulamasilile Gerceklistrilen Dusuk Guglu Laser Terapinin Yara Iyileşme Hizina

Musbahu Muhammad SANI

Biyomedical Muhendislik Programi
Yüksek Lisans Tezi

Danışman: Yrd. Doç. Dr. Haşim Özgür TABAKOĞLU

Düşük düzeyde lazer tedavisi iyileşme süreçlerinin tedavisi için önemli bir yöntemdir.Gruplar arasında iyileşme hızının yumruk yaranın üzerine darbeli LLLT'nin etkileri hemen yaralama sonra ışınlanmış ve bu yaralanma sonrasındaki sonra ışınlanmış histolojik ve morfometrik karşılaştırıldı. 12 Wister faresi hemen (iltihabi grubu) yaralama sonra (808nm den 5J/cm^2, 0.02W/cm2) lazer ışınlama dört gruba ayrıldı, 24 saat (proliferatif grubu) Yaralanma sonrası, 72 saat (proliferatif grubu) Yaralanma sonrası ve hiçbir ışınlama (Kontrol grubu). Hayvanlar kurban ve alınan doku örnekleri 3 histolojik analize tabi tutulmuştur, 7. ve 14. gün hedefimize izlemek Yaralanma sonrası. Tüm tedavi grupları için ortalama WHR testlerine göre, kontrol grubundan daha düşük ortalama değer ($p>0.05$) ile kontrol grubu arasında önemli bir farklılık olduğu bulunmuştur. Istatistiksel olarak anlamlı fark ($p>0.05$) sahip eski yüksek ortalama değere sahip proliferatif inflamatuar gruplar arasında bulundu. Proliferatif karşılaştırarak ve grupları yeniden üzerine, anlamlı bir fark çoğalma gruptan dayüksek ortalama değere sahip bulundu. Ancak eski olan biraz daha yüksek ortalama değere sahip şekillenmesi ve inflamatuvar gruplar arasında anlamlı bir fark yoktu. Elde edilen histolojik ve morfometrik sonuçlardan, granülasyon dokusu, fibroblastlar ve kollajen birikimi artış gösterdiği ilk olarak hızlı kontraktürünü sarılmış ve böylece şifalar önde gelen yaklaşık 24 saat sonrası yaralama (geç enflamatuar, erken proliferatif evre) uygulandığında LLLT en iyi etkiye sahip olduğu sonucuna.

Anahter Kelimeleri**:** LLLT, Inflammatuar, Çoğalma, Tadilat, Yara Iyileşmesi, Yara Daralma, GaAlAs

CHAPTER 1

INTRODUCTION

"A wound occurs when normal anatomic structure and function are disrupted by injury" (Lazarus et. al., 1994). The reparatory response is an innate defense mechanism of the host designed to restore structural integrity of the tissue, establish a physical barrier against infection, and return the damaged tissue to its normal state.

Health professionals and allies frequently tend for a variety of skin wounds including burns, ulcers, abrasions, and surgical incisions. From acute and chronic wound management to augmented scar remodeling, clinicians seek optimal methods of promoting wound healing, however this remains a challenge the world over.

Currently, skin injuries presents a dilemma of global proportions and attract great clinical interest due to high morbidity attributed to changes in the normal healing process (Snyder 2005). Economically, chronic wound management has been approximated to have reached a total cost of 2-4% of the health budget in western countries (Gottrup 2004) and that the annual expenditure on wound related problems in the USA alone exceeds one billion dollars (Ueno et. al., 2006). This led to the assertion that the estimate is expected to rise consequent upon of an increasing populace of the elderly, diabetic and obesity epidemic. Complications in non-healing wounds are vast, and patients are at risk of severe pain, septicaemia, hospitalization, and in some cases amputations (Trøstrup et. al., 2013). Additionally, these changes may also inflict increased cost to society, and reduce quality of life for a huge number of people worldwide.Presently, the search for more effective therapeutic strategies and cheap alternatives that might work in the healing process attracts much attention (Krishnan 2006). Much emphasis has been placed on tissue repair time in an effort to make the healing process fast, more harmonious with reduced complications in wound resolution.

This research is thus motivated by the necessity of enhancement of wound healing process which will lead to shorter stay in health care facility (reduced nosocomial infections), reduced cost of wound care, enhanced quality of life for patients with these wounds as well as faster return to routine activities.

Low level laser therapy (LLLT) is a form of phototherapy used to hasten wound healing under different clinical conditions. At an adequate wavelength, intensity, and dose, LLLT is thought to accelerate tissue repair. This led to its approval by the Food and Drug Administration (FDA) as an important method for treating healing processes (Nussbaum et. al., 1999). However, despite the development, there is conflicting information about the effect of multiple irradiations on the cellular responses of wounds.

LLLT aims at photo-activating cellular mechanisms, leading to reformation of the affected area by reducing edema, induction of analgesia, as well as accelerating the tissue repair process (Tumilty et. al., 2008). In accordance with Lins et. al., 2010, ‘’when laser light interacts with cells and tissues in an appropriate dosage, certain cell functions can be stimulated, among which are stimulation of lymphocytes, activation of mast cell, increase in mitochondrial ATP production and proliferation of several types of cells, thus promoting anti-inflammatory effects.

Studies showing that laser irradiation causes transformation of fibroblasts into myofibroblasts (Porreau-Schneider et. al., 1990) suggests the possibility of an increased rate of wound contraction in response to laser therapy. Thus in recent years, coherent light (laser) phototherapy has been used as biostimulator for tissue repair, as it helps to improve local circulation, cell proliferation and collagen synthesis (Vladimirov et. al., 2004, Desmet et. al., 2006 and Minatel et. al., 2009). Thus changes observed in low laser treated wounds include enhanced granulation tissue, epithelialization, fibroblast proliferation and matrix synthesis, and faster neovascularization.

Despite the well documentation of these cellular and biochemical events, application of the knowledge to wound healing has been frustrated by conflicting reports. The literature provides abundant contradictory data on low level laser biostimulation of wound healing. At present, the effect of different treatment interval is underexplored even though there is sufficient evidence to suggest that it is an important parameter (Huang et. al., 2009). Currently, wavelength, dosimetry, treatment schedule, and laser irradiation conditions are not well established. Thus this draws my attention to check the

effects of LLLT when applied immediately after wounding or later in the healing process.

Also there have been some conflicting reports in the literature. While some research works claim that LLLT has maximum effect when applied at earlier stage of wound healing (inflammatory phase) (Shumway 2007, Rezende et. al., 2007, Nunez et. al., 2012) others assert that maximum effectiveness is achieved only when it is applied at cellular stages (Walsh 1997,Eells et. al., 2004, Nazrul-Islam 2010).

1.1 Hypothesis

Being a new modality of inducing healing in wounds, several studies have been carried out in the search for a better understanding of the mechanisms of low level laser therapy (LLLT) and its therapeutic applications. However, several questions remain unanswered, and research on the optimal parameters of its use in the different stages of wound healing may help clarify these issues.

Therefore this study aims to check the effect of early and cellular (later) low level laser irradiation on wound healing rate using pulsed laser operating mode. We proposed that due to the differences in cellular properties of the tissue during each of the stages of wound healing, there is anticipated difference in response of each stage to LLLI. Thus in view of this, we wish to evaluate these differences by assessing our objectives (below).

1.2 Aim

The aim of this research work is to ascertain the effect of early and cellular (later) low level laser irradiation on wound healing rate using pulsed laser operating mode.

1.3 Objectives

The individual objectives of this research are;

1. To check wound contraction rate in all groups
2. To compare all groups morphometrically.

CHAPTER 2

2.1 Skin

Histologically, skin is divided into two functionally interdependent layers: epidermis and dermis of connective tissue (Murphy 2005). Below the dermis is a fatty layer, hypodermis, which in most mammals is separated from the rest of the body by a flat sheet of striated muscle (Rook et. al., 1986). Dermal flexibility and elasticity united with epidermal strength and impermeability allows for the protective function of the skin (Procsh et. al., 2008).

2.1.1 Epidermis

The epidermis, 80 μm (or about 0.1 mm) thick, is multicellular and has four sub-layers: the *stratum basalis*, the *stratum spinosum*, the *stratum granulosum* and the *stratum corneum* (Orgill 1983). New cells are continuously made in the deepest layer of the epidermis, the stratum basalis and move outward toward the surface of the skin (Mader 2004).

The majority of the epidermis is composed of mostly keratinocytes, while the others include small subpopulations of melanocytes, Langerhans cells, mechanoreceptor neuroendocrine (Merkel) cells and unmyelinated axons and lymphocytes that engage in immunologic protection (Fawcett, 1986). Tight intercellular connections within the epidermis form the basis of its physical integrity, and lamellar granules, containingsterols, polar lipids and hydrolytic enzymes, released into the intercellular space, give the epidermis its impermeable quality (Landmann, 1986 and Paletta et. al., 2006).

Keratinocytes produce several protective immunogenic molecules (Steinhoff et. al., 2001) including interleukins (IL-1, IL-6, IL-8), interferons (IFN-α, IFN-β), transforming growth factors (TGF-α, TGF-β), fibroblast growth factor (FGF), platelet-derived growth

factor (PDGF), tumor necrosis factor (TNF-α), , and granulocyte-macrophage colony-stimulating factor (GM-CSF or G-CSF).

Wounds involving only the epidermis heal by regeneration of epidermal cells not only from the wound's periphery but also from skin adnexal structures, including sweat and sebaceous glands, and hair follicles. Given the ability of the epidermis to regenerate, pure epidermal wounds heal without scarring (Paletta et. al., 2006).

2.1.2 Dermis

This is a dense fibro-elastic connective tissue of 1-4 mm thickness (Odland 1991), responsible for the mechanical properties of skin (Orgill 1983). Anatomically separated into two, the superficial papillary dermis and the deep reticular dermis, it consists of fibers of connective tissue made up of ground substance and elastin and collagen that run in all directions. Collagen and elastin fibers are both embedded in an inter-fibrillar matrix of proteoglycans. The latter provide recoil strength while the former provide strength to the skin (Scanlon & Sanders 2006).

The dermis contains an extensive plexus of blood vessels that provides nourishment for itself and the epidermis. Dermal microvessels are surrounded by a complement of immune cells, including neutrophils, macrophages, mast and dendritic cells. Collectively, these cells collaborate to coordinate intra-dermal antigen presentation, induction of inflammation and homeostasis in the immediate perivascular microenvironment (Murphy et. al., 2005).

The dermis being a complex, dynamic microenvironment that harbors a collection of specialized cells within an intricate matrix (formed by soluble and non-soluble molecules), in a normal physiological state contains an ensemble of cells that includes; fibroblasts, endothelial cells, monocytes/macrophages, dendritic cells, mast cells, lymphocytes, Schwann cells, axons and specialized nerve endings, pericytes and glomus cells and mesenchymal elements with presumed differentiation plasticity (mesenchymal stem cells) (Orgill & Blanco 2009). These cells are contained within an acellular matrix, composed mainly of collagen and glycosaminoglycan.

Collagen comprises almost 75% of fat free dry weight and 18-30% of the dermal volume (Ebling et. al., 1992). Finlay (1969) showed that bundles of collagen fiber form

an irregular network that runs almost parallel to the epidermal surface. Interwoven among the bundles of collagen, is a network of elastin that restores the normal fibrous array following its deformation by external mechanical forces. According to Oxlund et. al., (1988), there is no direct connection shown between elastin and collagen fibers, but collagen fibrils appear to wind around the elastin cores. Directly beneath the undersurface of the epidermis lies a basement membrane mostly composed of collagen IV, which physically separates epidermal from the dermal layers.

2.2 Wound Healing

This is the complex biologic and biochemical process which starts immediately upon implication of an injury and responsiveness of its cellular components to light energy stimulation has been studied (Hemvani et. al., 1998 and Yu et. al., 1996). A rise in cellular energy, oxygenation, enhanced micro-circulation and synthesis of specialized signaling proteins, have been shown to be caused by photons - the reason for acceleration of wound healing.

Normally, healing occurs by an overlap of phases of dynamic tissue reactions including proliferation and migration of cells and proliferation of soluble factors such as growth factors (GFs) and cytokines, matrix components, and synthesis of elements of extracellular matrix, including collagen, elastic and reticular fibers (Pugliese et. al., 2003) acting in concert to repair tissue damage (Keswani & Crobleholme 2005).

Three stages of wound healing with distinct biochemical profiles leading to restoration of tissue continuity and function have been described. Stage 1, hemostasis and inflammation (0 to 4 days), are followed by stage 2, proliferation (3rd day to 3 weeks) and stage 3, maturation (3 weeks to 2 years) (Scheithauer & Riechelmann 2003).

2.2.1 Homeostasis & Inflammation

This is the initial response to injury involving transient 5- to 10-minutes of intense vasoconstriction, aiding hemostasis, superseded by vasodilation that mostly peaks about 20 minutes post injury and is followed by a rise in capillary permeability (Stadelman et. al., 1998). Platelets aggregate at the injury site to degranulate, thereby initiating the clotting cascade. Within minutes, hemostasis is achieved by forming a fibrin clot.

Within 24 hours, neutrophils arrive at the wound site, ushering in the inflammatory phase (which lasts 3-4 days) (Diegelmann & Evans 2004). This is facilitated through a variety of chemical signaling mechanisms, including the complement cascade, activation interleukins and signaling of transforming growth factor-β (TGF-β), leading to passage of neutrophils down a chemical gradient towards the wound, termed chemotaxis (Broughton et. al., 2006). Their main function is not phagocytosis, but the enzymatic destruction of fibrin. Soon after that, monocytes appear which will turn into macrophages on approximately the fifth day.

Macrophages are phagocytic cells which reach peak concentration in a wound about 48-72 hours after injury. They survive the more acidic wound environment and harbors large reservoir of growth factors, including TGF- β and epidermal growth factor (EGF), which are important regulators of inflammatory response, stimulating angiogenesis and enhancing granulation tissue formation (Velnar et. al., 2009).

A multitude of factors, such as lipoxins and products of arachidonic acid metabolism, are thought to have anti-inflammatory properties which subjugate the immune response and usher-in the next phase of wound healing (Nathan 2009).

2.2.2 Proliferation

In two to three days post wounding, fibroblasts start to appear in the wound, marking onset of the proliferative stage even before the completion of the inflammatory stage (Falanga 2005). As with the other stages of the healing process, steps in this stage do not occur in series but rather partially overlap in time with characteristic angiogenesis, deposition of collagen, formation of granulation tissue, epithelialization and contraction of the wound (Midwood et. al., 2004 and Kuwahara and Rasberry 2007).

Additionally, formation of a fibrin, fibronectin glycosaminoglycan, and hyaluronic acid matrix which, initially, is populated with platelets and macrophages also marks the beginning of this stage. Macrophages secretion of various GFs enhances fibroplasia, and fibroblasts migrate into the wound using the fibrin and fibronectin matrix as a scaffold. In response to these GFs, fibroblasts proliferate and dominate the wound by third to fifth day post injury (Richard et. al., Nd).

Fibroblast proliferation is stimulated by growth factors released from the haemostatic clot which then migrate to the wound (predominantly by TGF-β and PDGF). This

occurs, concurrently with angiogenesis, when endothelial cells migrate to the wounded area (Kuwahara and Rasberry 2007).By the third day, fibroblasts becomes enough to lay down extracellular matrix proteins (hyaluronan, fibronectins and proteoglycans) and subsequently produce collagen and fibronectin. A resultant pink, vascular, fibrous granulation tissue replaces the clot at the site of a wound (Young and McEllen 2011).

Hypoxia causes the release of vascular endothelial growth factor (VEGF) which, in combination with other cytokines, induces endothelial cells to trigger repair of damaged blood vessels and neovascularization (Young and McEllen 2011). This new vasculature allows nutrients delivery and cellular metabolic waste removal. Granulation tissue, a dense population of blood vessels, macrophages, and fibroblasts with a loose connective tissue matrix (Lorenz & Longaker 2003), may accompany the process in wound healing by secondary intention.

Epithelial mesenchymal transition (EMT), allows epithelial cells to become motile and travel across the wound surface (Yang and Weinberg 2008). This is caused by changes in cytokine concentration which transforms the cells from motile phenotypes to proliferative ones thus repopulating epithelial cell levels and completing wound repair (De Donatis 2010).

Collagen, a triple helical structured molecule with abundance of hydroxyproline and hydroxylysine, is the predominant ECM protein deposited at the wound site. A process of hydroxylation that forms these two amino acids requires ascorbic acid (vitamin C) and is necessary for stabilizing and cross-linking the collagen molecule (Fine & Mustoe 2001). During the initial healing process, there is an abundance of type III collagen, composed of thin fibrils and is relatively pliable. Type I collagen is also formed, and with remodeling, it becomes the most abundant form found in normal adult wounds with a 4:1 ratio to type III collagen. Type I collagen is relatively rigid and imparts high tensile strength to the tissue (Keswani & Crobleholme 2005b).

This process correlates in time with phenotypic morphogenesis of fibroblasts into myofibroblasts which appear in the wound about 4 to 6 days after injury and are identified by their contractile properties, attributed to increased amount of actin filaments, as demonstrated by electron microscopy. Myofibroblasts facilitate wound contraction in about 7 days post injury. Actin and myosin interactions pull the cells closer together thereby decreasing wound area. This can occur at a rate of 0.6-0.75

mm/day leading to shortened scars (Lawrence 1998). Factors influencing the contraction include wound shape, with linear wounds contracting fastest and circular wounds the slowest. Abnormalities in this stage of healing can lead to contractures and even deformities (Hinz 2006).

2.2.3 Remodeling

Accumulation of collagen in the wound peaks at 2–3 weeks after injury, after which the rate of its synthesis declines (Monaco and Lawrence 2003) signaling transition to remodeling. A balance between synthesis, deposition, and degradation is thus achieved in this stage. Wound tensile strength increases as the initial randomly deposited collagen fibrils are replaced by organized more cross-linked ones. The enzyme lysyl oxidase ensures cross-linking of these fibrils. The normal adult 4:1 ratio of type I to type III collagen is restored during this stage. A balance between collagen deposition and degradation is established in part by degradation of ECM components by stromelysins, Matrix metalloproteinases (MMPs), collagenases, and gelatinases (Soo et. al., 2000).

2.3 Scarring

Denoting absence of tissue organization compared to surrounding normal tissue, a scar is, morphologically, characterized by disorganized collagen deposition. This fibrotic disorder results from a disruption in the normal process of healing (Sarrazy et. al., 2011). Epithelial and ECM changes seem to be involved in excessive scarring (Dabiri et. al., 2008). Furthermore, hypertrophic scars formed due to inhibition of apoptosis in wounds mechanically irritated early into proliferative phase have been observed (Aarabi et. al., 2007).

There are reports corroborating that inflammation during the healing process is directly linked to the extent of scar formation (Eming et. al., 2007). First fact is that fetal wound healing, which typically lacks inflammatory response, is scarless up to a certain age (Redd et. al., 2004 and Bullard et. al., 2003). Additionally, induction of inflammation in fetal wounds tends to extend scar formation (Whitby and Ferguson 1991).

During wound maturation, ECM components undergo certain changes. Collagen I oriented in small parallel bundles and, therefore, different from the basket-weave collagen in healthy dermis replaces Collagen III, produced in the proliferative phase (Gurtner and Evans 2000). Later on myofibroblasts cause wound retraction through

multiple attachments to collagen and thus decrease the area developing scar (Tziotzios et. al., 2012 and Profyris et. al., 2012). Finally, there is diminishing of angiogenic processes, decline in flow of blood to the wound, and slowing and eventual stoppage of metabolic activity in the wound.

2.4 Biochemistry of Mitochondrial Cytochrome C Oxidase

Current studies into mechanism of LLLT effects inevitably involve mitochondria, a key organelle of energy generation and metabolism via oxidative phosphorylation. Low level laser therapy (LLLT) activates processes that intensify cellular physiologic activity. Several studies revealed the potentials of LLLT to stimulate the mitochondrial respiratory chain. It is believed that cytochrome c oxidase, a mobile enzyme transferring electrons between complexes III and IV of the electron transport chain, is the component targeted by near-infrared light stimulation (Karu et. al., 1995, Afnasyeva et. al., 2005 and Pastore et. al., 2000). Absorption spectra for this enzyme recorded at different oxidation states were found to be similar to that for biological responses to light (Karu and Kolyakov 2005). This observation led to the proposal of cytochrome c oxidase as being the photoacceptor for the red to NIR range in mammalian cells (Karu and Kolyakov 2005).

It is thus believed that stimulation of Cox leads to accelerated transfer of electrons and up-regulation of oxidative phosphorylation, and thus production of more ATP molecules (Wong-Riley et. al. 2001 and Wong- Riley et. al. 2005). Recent developments also indicate that under physiological conditions nitric oxide regulate cytochrome c oxidase activity via reversible inhibition of mitochondrial respiration (Brown 1999).

2.5 Mechanism Of LLLT

A photobiological law states that “for low power visible light to be of effect on living biological system, photons must be absorbed by electronic absorption bands of some molecular photoacceptors, or chromophores (molecules which occur either as conjugated pi electron systems or metal complexes, and defines the color of the compound of which it is an ingredient)” (Sutherland 2002).

The cellular mechanism of LLLT has been attributed to monochromatic visible and NIR radiation absorption by components of electron transport chain (Karu 1989). Evidences

suggested mitochondria to be responsible for cellular response to red and NIR light. Illumination effects on isolated rat liver mitochondria included increased proton electrochemical potential, increased ATP, NADH, RNA and protein synthesis (Passarella et. al. 1984 and Greco et. al., 1989) and increase in membrane potential, and oxygen consumption.

The results of various studies (Karu et. al., 1998, Karu et. al., 2001a, Karu et. al., 2001b, and Kolyakov et. al., 2001) support that cellular mechanism of low power laser therapy being based on increased mitochondrial oxidative metabolism, caused by excitation of electronic components of the respiratory chain.

Cellular manifests of LLLT have been classified into primary, secondary, and tertiary (Schindi et. al., 1999; Stadler et. al., 2004). Generally, Primary reactions refer to reactions due to photon absorption while secondary effects have no peculiarity to LLLT and, as their occurrence depended on cell sensitivity, are not as certain as the primary. Tertiary (systemic) effects are the least predictable as they are affected by both internal and external environment and by intracellular interactions which explain why the treatment of one lesion can also stimulate healing in other lesions present (Dyson, 2006).

2.5.1 Primary Mechanisms of Light Action

Laser emitted photons fall upon mitochondria (Theralase 2003) and cell membranes of fibroblasts, keratinocytes and endothelial cells, where chromophores (cytochromes, porphyrins and flavoproteins) absorb and convert the heat energy to chemical kinetic energy (Matic et. al., 2003 and Olsen et. al., 1980) within the cell. This changes membrane permeability improves mitochondrial signaling with nucleus and cytosol, nitric oxide formation and increases oxidative metabolism thereby producing more ATP (Morimoto et. al., 1994; relief from Yu et. al., 1997 and Karu, 1989), which further results normalization of cell function, pain and healing of wound (Matic et. al., 2003; Dyson, 2006; Olsen et. al., 1980). Some hypotheses proposed to explain LLLT mechanism of include:

2.5.1.1 Singlet Oxygen Hypothesis

This hypothesis was put forward based on visible laser light action on RNA synthesis rates in HeLa cells and spectroscopic data for porphyrins and flavins, (photo-absorbing

molecules that can reversibly be converted to photosensitizers) (Giese 1980), that "the absorption of light quanta by these molecules was responsible for the generation of singlet oxygen $^1O_2$ and, therefore, for stimulation of RNA and DNA synthesis" (Karu et. al. 1981b and Karu et. al., 1982).

2.5.1.2 NO Hypothesis

Mitochondrial nitric oxide can bind to Cox, displacing oxygen competitively, inhibiting respiration especially in hypoxic or stressed cells (Brown 2001). Also NO concentration is increased under pathological conditions (resulting from activation of NO producing macrophages (Hothersall 1997). It was hypothesized (Karu 2000 and Lane 2006) that "laser irradiation and activation of electron flow in cytochrome c oxidase molecule could reverse the partial inhibition of the catalytic center by NO and thus increase binding of oxygen and respiratory rate". Increased concentrations of nitric oxide have been observed in cell cultures and animals after LLLT due to its release, from Cox in the mitochondria and from other intracellular stores such as nitrosylated hemoglobin and myoglobin, by the photon energy (Shiva and Gladwin 2009).

2.5.1.3 Redox Properties Alteration and Superoxide Anion Hypothesis

The redox state of certain chromophores (such as CuA and B or hemes *a* and *a3*) in cytochrome c oxidase molecule and, consequently, the rate of electron flow in the molecule are affected by photo-excitation (Karu et. al., 1995). It was thus proposed that low or no cellular response is observed at overall optimal cellular redox potential for the particular growth conditions. However, there is stronger response in more reduced cellular redox potential. This may explain the disparity of cellular responses and sometimes the nonexistence in different experiments. A rise in intracellular pH due to irradiation has been measured experimentally both in mammalian cells (Chop et. al., 1990) and in *E. coli* (Quickden et. al.,1995).

Reactive oxygen species (ROS) formed as natural by-products of oxygen metabolism, have crucial roles in cellular signaling (Storz 2007), regulating nucleic acids and protein synthesis, activation of enzymes and progression of cell cycle (Brondon et. al., 2005). In 1993, Karu et. al., suggested that "activation of the respiratory chain by irradiation would also increase production of superoxide anions, the production of which depends primarily on the metabolic state of the mitochondria" (Forman and Boveris 1982).

It was reported that LLLT shifts the overall cellular redox potential in favor of greater oxidation (Karu 1999) and increased generation of ROS and cell redox activity have been demonstrated (Chen et. al. 2009b; Pal et. al. 2007; and Zhang et. al.2008). These cytosolic changes may subsequently induce transcriptional changes (regulation of transcription factors such as nuclear factor β NF-β).

2.5.1.4 Local Transient Heating Hypothesis

Upon exciting electronic states with light, part of the energy is converted to heat, thus causing a local transient rise in the temperature of the absorbing molecules (Karu et. al., 1991a). This transient increase in temperature of the biomolecules may in turn cause structural or conformational changes and trigger biochemical activities (cellular signaling or secondary reactions) (Karu et. al., 1991b and Karu et. al., 1992).

Interestingly, Tiina 2003b falsified the assertion that only one of above reactions occurs upon irradiating and formation of excited electronic states in cells, stating that "it is entirely possible that all the mechanisms proposed above lead to modulation of the redox state of the mitochondria (a shift in the direction of greater oxidation)". However, depending on dose and intensity of the light used, some of these mechanisms may significantly prevail. Experiments with *E. coli* showed that, different mechanisms take place at different laser doses, with photochemical at low doses and thermal at higher doses (Karu et. al., 1994).

2.5.2 Secondary (Cellular Signaling) Reactions

The above primary photoreactions are amplified by cellular signaling. A metabolic cascade at cellular level results in various physiological changes including cell membrane permeability (Dyson 2006 and Smith 1991). Mitochondrial calcium is released into the cytoplasm thereby changing intracellular calcium levels (Tunér and Hode, 2002) and thus stimulating cell metabolism and regulation of signaling pathways responsible for processes required for wound repair such as migration of cells, synthesis of RNA and DNA, cell mitosis, secretion of proteins and proliferation of cells (Takac and Stojanovic, 1998; Kiepeis et. al., 2001). All these events are mediated through cascades of cellular signaling and photosignal transduction (Tiina2003).

Studies revealed that intracellular redox state regulates many cellular signaling pathways (Gius et. al., 1999, and Kamata & Hirata 1999). It is believed that

extracellular stimuli elicit cellular responses such as proliferation, differentiation, and even apoptosis through cellular signaling pathways. Cellular redox state modulation also affects gene expression through cell signaling mechanisms (via effector molecules like transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein (AP)-1 (Gius et. al., 1999), and phospholipase A_2) (Sun & Oberley 1996, Nakamura et. al., 1997). Generally, cell signaling systems are activated by oxidants, while reductants inhibit upstream signaling cascades, with resultant suppression of transcription factors (Calkhoven and Geert 1996). Despite similarity in cellular signaling, the resultant cellular responses to irradiation may differ due to variation in modes of regulating transcription factors.

An attempt was made to quantify the magnitude of irradiation effects as dependent on the metabolic status of *E. coli* cells (Tiphlova and Karu 1991). A correlation was found between the amount of ATP in irradiated cells and the initial amount of ATP in control cells (Karu et. al. 2001). Thus, variations in the effects of low level laser at the cellular level are explained by the cellular redox state (and pHi) at the time of irradiation.

2.5.3 Tertiary Response

These are effects induced in cells away from site of secondary reactions (Dyson, 2006). Communication between irradiated and with non-irradiated cells, occur through increased levels of cytokines or growth factors (Vladimirovlu 2004 and Tunér and Hode, 2002b). This enhances immune response by activating T-cells, macrophages and mast cells, with subsequent increased synthesis of endorphins and decrease in bradykinin resulting in pain relief. (Hamblin and Deidova, 2006).

2.5.4 Metabolic Enhancement via Non-mitochondrial Chromophores

Mechanism of regulation of redox activities cannot occur via the respiratory chain alone. Redox chains containing visible spectra chromophores are most at times key structures of regulating metabolic pathways. NADPH-oxidase multi-component enzyme system of phagocytic cells, responsible for non-mitochondrial respiratory burst, is a redox chain that generates reactive oxygen species in response to microbicidal or other types of activation. Irradiation with the He-Ne (Karu et. al., 1989 and Karu et. al., 1997) and semiconductor lasers and LEDs (Karu et. al., 1993b, Karu et. al., 1993) can activate this chain.

CHAPTER 3

LITERATURE REVIEW

3.1 Low Level Laser Therapy (LLLT)

Low level laser therapy at cellular level causes alterations biochemically and bioenergetically, leading to metabolic enhancement, proliferation and maturation of cell, increased quantity of granulation tissue with decreased inflammatory mediators and enhanced healing process (Silva et. al., 2007 and Bourguignon et. al., 2005). Furthermore, cellular absorption of laser irradiation results in increased cell metabolism with characteristic stimulation of mitochondrial respiratory chain photoreceptors, changes in cellular level of ATP, release of growth factors, and synthesis of collagen (Postain et. al., 2005).

Unlike high energy tissue vaporizing lasers, low energy laser stimulation of tissue has no thermal effects. These lasers emit visible spectra of 630–640 nm that penetrates dermal layers (Eells et. al., 2004 and Bortoletto et. al., 2004). Replication of DNA, proliferation of cells, enhancement of microcirculation and other cellular processes have been shown to occur between the red and IR wavelength. Low laser irradiation has also increased growth factors, with subsequent angiogenesis, release of cytokine and increased production of cellular matrix (Burd et. al., 2005 and Maiya et. al., 2005).

Both *in-vitro* and *in-vivo* experimental studies on LLLT have been underway since 1960s, which led to its approval by U.S. Food and Drug Administration as an important method for hastening wound healing in early 1990s (Sugrue et. al., 1990, Chromey 1992 and Nussbaun et. al., 1994).

Low doses of laser irradiation have been shown to stimulate in vitro proliferation of fibroblasts (Lubart et. al., 1992; Yu et. al., 1994), keratinocytes (Grossman et. al., 1998),

endothelial cells (Moore et. al., 2005), and lymphocytes (Stadler et. al., 2000). The proposed proliferative mechanisms involve photo-stimulation of mitochondrial processes, which enhance release of growth factor, ultimately leading to proliferation of cells (Bjordal et. al., 2007). In 2003, Kreisler et. al., showed a dose dependent enhancement of human gingival fibroblasts proliferation by low level irradiation. Shefer et. al. 2002, showed that low laser irradiation could activate skeletal muscle satellite cells, enhance their proliferation, inhibit differentiation and regulate synthesis of proteins. Furthermore, modulation of matrix metalloproteinase activity and gene expression in porcine aortic smooth muscle cells were evident (Gavish et. al., 2006).

Many reports from both animal model and clinical studies demonstrated the beneficial effects of LLLT on various diseases and injuries, and its usage in treating both chronic and acute conditions. LLLT enhances neovascularization, stimulate angiogenesis and increase synthesis of collagen to promote healing of acute (Hopkins et. al., 2004) and chronic wounds (Yu et. al., 1997). It also accelerates healing of cutaneous wounds in rats with a biphasic dose response in favor of low doses (Corazza et. al., 2007).

Furthermore, positive randomized clinical trials have been published on diverse pathologies including osteoarthritis (Ozdemir et. al., 2001), tendonopathy (Stergioulas et. al. 2008), wounds (Caetano et. al., 2009; Ozcelik et. al., 2008; Schubert et. al., 2007), back and neck pain (Basford et. al., 1999 and Chow et. al., 2006), muscle fatigue (Leal Junior et. al., 2008), peripheral nerve injuries (Rochkind et. al., 2007) and stroke (Zivin et. al., 2009).

Nevertheless there have been negative results observed under certain circumstances. This may be due to improper dosimetry, treating anatomically inappropriate location, concurrence with medication (such as steroidal and non-steroidal anti-inflammatories which can inhibit healing) and other factors (Goncalves et. al., 2007).

Many medical centers have been using this modality in treating wounds for over two decades (Postin et. al. 2005, and Adeir et. al. 2007). However, despite the vast clinical usage, controversy lingers regarding its efficacy in wound treatment (Basford 1995, Baxter 1997, and Turner & Hord 1998). Many laser systems, (Houreld & Abrahamse 2007), different laser parameters, (Mendez et. al. 2004), varied irradiation protocols (frequency, exposure time, and duration of treatment) (Hawkins & Abrahamse 2006) and a variety of treatment modalities led to these conclusions (Franek et. al., 2002).

Lasers have been shown to be stimulatory, inhibitory, and or not effective at all. These observed differences may be explained by the differences in treatment parameters used (Baxter et. al., 1991). Karu (1987) made an interesting statement that "the magnitude of the laser biostimulation effect depends on the physiological state of the cell at the moment of irradiation". This may explain the undetectability, as well as variability of results reported in the literature. Currently, the lack of single accepted actual explanation of mechanism of low level laser biostimulation complicates the evaluation of the conflicting reports in literature.

A study on the effect of LLLT on wound healing by Medrado et. al., 2003, led to the conclusion that effectiveness of low laser therapy led to reduction of inflammatory reaction and increase in collagen deposition with a greater myofibroblast proliferation in cutaneous experimental wounds. (Medrado et. al., 2003). Furthermore, to ascertain the behavior of skin wounds caused in dorsal region of rats, Rocha et. al., used a low level laser with a dose of 3.8 J/cm^2, power of 15mW and exposure time of 15 seconds and noticed increased neovascularization and proliferation of fibroblast as well as reduction in inflammatory infiltrate in the wounds submitted to laser therapy (Rocha et. al., 2006).

In a study aimed at investigating cellular, structural and molecular responses of wounds to 632.8 nm He-Ne laser irradiation, Hawkins & Abrahamse applied a single dosage of 0.5, 2.5, 5, or 10 J/cm^2 on normal and injured human skin fibroblasts. They used light microscopy to evaluate morphological changes in the two fibroblasts. Cellular evaluation parameters included cell proliferation and viability, and cytotoxicity while molecular parameter assessed magnitude of damage to DNA. The results obtained demonstrated the effect of LLLT on the normal and wounded fibroblasts cells. Observation showed that doses of 0.5, 2.5, 5, and 10 J/cm^2 produced measurable changes in fibroblast cells. Thus they concluded that a dose of 10 J/cm^2 appeared to causes significant cellular and molecular damage, and should be considered for other therapies, such as photodynamic therapy (Hawkins & Abrahamse 2005).

In a related study, Hawkins & Abrahamse tried to establish the response of wounded human skin fibroblasts to 632.8 nm He-Ne laser irradiation using one, two, or three exposures at 2.5, 5.0, or 16.0 J/cm^2 on each day for 2 days consecutively. In the methodology, cellular responses to the laser irradiation were evaluated by measuring changes in cellular proliferation, morphology and viability, and damage due to multiple irradiations. Their results revealed that a single 5.0 J/cm^2 dosage and two or three 2.5

J/cm^2 doses had stimulatory effect on wounded fibroblasts with an increase in cellular proliferation, migration and viability, but without inflicting stress or damage to the cells. However, they found that multiple exposures to higher doses (16 J/cm^2) caused additional stress, thereby reducing ATP activity, cellular migration, viability, and inhibits proliferation.

In conclusion, their results showed that right fluence (J/cm^2) and number of exposures can stimulate cellular response in wounded fibroblasts and promote cellular migration and proliferation enhancing mitochondrial activity and maintaining viability without stressing cells. The results further suggest that the cumulative effect of lower doses (2.5 or 5 J/cm^2) of laser irradiation determines the stimulatory effect, while multiple exposures at higher doses (16 J/cm^2) result in an inhibition and some damage. (Hawkins & Abrahamse 2006).

Numerous related studies showed that low level laser irradiation led to activation of fibroblasts and keratinocytes, a critical factor in wound healing. In their research on the modulating proliferation of fibroblast and inflammatory response by low level laser therapy in tissue repair process, Moriera et. al., subjected wounded wister rats (n=6) to LILT (0hrs, 48hrs and 7days post surgery) at 3.8 J/cm^2 dosage, 15mW potency and duration of 15seconds. Histomorphometric analysis at 10 days post-surgery demonstrated that animals treated with LILT presented a greater number of fibroblasts per microscopic field. Also in LLLT group, histopathological studies evidenced material showed undamaged epidermis covering developed granulation tissue, with organized parallel oriented collagen fibers, signifying organized healing process (Moriera et. al., 2006).

To evaluate the systemic effects of laser irradiation, two parallel incisions were made and sutured on the back of rats. After surgery, one wound on each rat received LLLT irradiation at 670nm and fluence of 30 J/cm^2 daily while the second one served as control receiving no irradiation. Wound evaluation followed at 24, 48, 72, 96, 120, 144, and 168 hours post operation. On comparison of irradiated with non-irradiated wounds, accelerated inflammatory as well as the proliferative and remodeling phases were observed in laser stimulated wounds. Regeneration of damaged epidermis and striated muscle repair were also stimulated (Gal et. al., 2006).

In their research, Krynicka et. al., tried to check if laser biostimulation starting on the first day after surgery, of brachial plexus or peripheral nerves, has therapeutic effect on the healing process. They divided 44 wistar rats into control group 1, where the wounds were allowed to heal spontaneously, and an experimental Group 2, where the wounds received laser irradiation with 810 nm, 100 mW, energy 15 J, exposure surface 3 cm^2, and 2 min. 30 sec. single application time, using CW mode. They assessed outcomes pathomorphologically (gross wound appearance), light and electron microscope studies, and tensile strength examination. The gross and microscopic findings indicated that low energy infrared laser radiation is of beneficial effect to the scar covering, with stratified squamous cornifying epithelium and intensified healing process. This led to their conclusion that laser stimulation is of beneficial effect on healing process. Furthermore, the findings underscored the utilization of biostimulative lasers early post-operation. However, physicomechanical test reveal no effect of infrared laser biostimulation on tensile strength of the cutaneous scar (Krynicka et. al., 2010).

In a study to investigate effects of phototherapy on nitric oxide and collagen production early into wound healing, Lanzafame et. al., created four full thickness 1cm incisions on the dorsum of rats. They inserted two sterile 8.0mm PVA sponge discs beneath two sides of each wound, and subsequently approximating with a 6-0 Prolene sutures. Each group (n=3) was irradiated with either 670nm, 728nm or 880nm daily on 0 to 5^{th} day post injury using NASA LEDs at fluences of 700mW/cm^2 and a dose of 7J/cm^2 per session. The animals were sacrificed on 7th day post wounding. Blood samples were obtained and sponges excised and submitted to histological evaluation (of the wounds) after hematoxylin eosin staining. Their results demonstrated that single wavelength near infrared phototherapy is beneficial during earlier phase of the healing process. They thus recommended further investigation into the effects of using combined wavelengths and therapy applied later into the phases of the healing process. (Lanzafame et. al., Nd).

Rezende et. al., 2007, studied the effect of a single laser irradiation on the healing process of full thickness skin lesions in rats. They subjected three groups of forty-eight male rats to 8mm diameter punch surgical lesion on the back. The control group received no treatment after surgery, while the other two groups received either 1.3 J/cm^2 or 3 J/cm^2energy doses from an 830-nm near-infrared diode laser immediately post wounding, with a constant intensity of 53mW/cm^2 for both groups. Biometrical and histological analyses were undertaken on 3, 7 and 14 days post wounding. Their results

revealed that irradiated lesions presented a more advanced healing process than control group. Additionally, a dose of 1.3 J/cm^2 led to better results as lesions in the group that received such presented faster lesion retraction with quicker re-epithelization and reformation of more organized collagen fibers (Rezende et. al., 2007).

In a related study, Nunez et al., 2012, in a study to check whether single dosage of a red laser (660 nm) differs from fractionated irradiation protocol in full thickness burns, subjected 36 rats to two lesions. Fractionated dose group received 1J/cm^2 on 1st, 3rd, and 10th days post wounding, the single dose group received 4 J/cm^2 immediately after wounding, while the control group was not irradiated. Using Doppler flowmetry, blood flow on specific days was measured. They later killed the animals and using HE dye, subjected them to differential leukocyte counting and angiogenesis. They observed that on day 21, CG group exhibited new vessel peak, FG group exhibited a significant number of cumulative neutrophils (with new vessel peak at day 15) while the SG group showed greater number of mononuclear cells. Thus they concluded that both irradiation protocols used accelerate angiogenesis and chemotaxis in burn wounds, suggesting single LLLT dose acceleration of inflammatory phase of wound repair (Núñez et. al., 2012).

In a study, Osman et. al., 2008, tried to evaluate the effect of an 808nm diode laser on the tissue healing process in steroid immunosuppressed rats. Forty eight Albino rats were exposed to immunosuppression via steroid injection and then divided into two groups, the first serving as control group while the other was exposed to diode laser 808 nm, 100mw, in defocused mode for 240 seconds. Rats were sacrificed after 3, 5, 7 and 10 days. They grossly observed faster wound closure with scarless healing in treated group than control which was confirmed by histopathological studies using hematoxylin and eosin and Masson's Trichrome stain. This revealed a moderate inflammatory reaction in the treated group versus severe reaction in the control group. Laser group also showed earlier granulation tissue formation and re-epithelization than control group. They thus concluded that LLLT using diode laser 808nm accelerated healing process even in immunosuppressed animal model with no scar formation (Osman et. al., 2008).

In a research to investigate the effect of pulsed low level laser irradiation on healing of third-degree burn in rat, Ezzati et. al., 2009, created two third degree burns, distally and proximally, on the skin of 74 rats divided into four groups. The proximal burn served as

control in of all groups. In the first group, the distal burn was exposed to laser switched off while second and third groups were treated with 3,000 Hz pulsed infrared diode laser (890nm) with 2.3 and 11.7 J/cm^2 fluences, respectively. The 4th group received topical treatment with 0.2% nitrofurazone. Both microbiological and macroscopic assessment of response to treatment and chi-square test result showed significant decrease incidence of *Staphylococcus epidermidis*, *Lactobacillus*, and diphtheria in irradiated groups compared with other groups. Independent sample *t*-test analysis also revealed that irradiation with 11.7 J/cm^2 fluence significantly increased rate of wound contraction at 3 and 4 weeks after burning compared with control burns (p = 0.018 and p = 0.01, respectively). They thus concluded that pulsed mode LLLT with 11.7 J/cm^2/890 nm on a third degree burn in rat model significantly increased rate of wound contraction when compared with control burns.

In a study aimed at facilitating and accelerating healing and wound contraction with reduced scar in an open wound, Hussein et. al., 2011, divided 20 adult male lepus cuniculus demostica rabbits into control group1 and treatment group 2. After anaesthesial administration, animals were subjected to 4cm length and 3cm depth wound on the gluteal region. The wounds in treatment group were irradiated with pulsed GaAlAs diode laser 890nm with output power of 10mW and 20 KHz frequency for 5 minutes six hours post operation. This was repeated for a week.

Histopathological findings of control group on 3rd day post operation revealed hemorrhage and clogged blood vessels in the gap with neutrophils infiltration. On the 7th day, there were neutrophils with hemolysis and granulation tissue in the gap under the dermis. On the 14th day, proliferating irregular fibrous connective tissue with mononuclear cell infiltration was observed. On the other hand, treatment group 2 results on the 3rd day showed severe neutrophils infiltration with fibroblasts proliferation from a few fibrous connective tissues. On 7th day, the lesion was characterised by severe granulation tissue consisting of proliferative fibrous tissue and mononuclear cell infiltration.

On day 14, the dermal sections showed debris material surrounded by dense collagen connective tissue with less fibrous tissue. Complete wound and skin layer closure was observed with less scar formation and shrinking of wound size up to 2cm unlike in group 1 where there was no complete wound closure and noticeable shrinkage in size.

Hussein et. al., thus concluded on the effectiveness of LLLT in open wounds with better regeneration, faster healing and restoration of structural integrity (Hussein et. al., 2011).

In another study to check the effect of LLLT on healing of skin incisions made using a diode laser in rats, Güngörmus and Akyol 2009 subjected eighteen wistar rats (divided into two groups: Group 1, diode laser control; Group 2, diode laser + irradiation) to two parallel incisions about 15mm long on either side of the dorsum using a diode laser (635-nm, power output 4W, 300 mm diameter tip, and 6mm long). The wound on the left side of each rat was irradiated at (fluence 10 J/cm^2, power density 0.1W/cm^2 from an 808-nm GaAlAs laser, CW) starting immediately post surgery and repeated on the 2nd, 4th, 6th, and 8th days (5 sessions) post surgery with 10 J/cm^2 (total dose) applied 20 s (2 J/cm^2) per session (total 100 s) within a 1 cm^2 biostimulation spot and approximately 1 cm from the wound surface.

They sacrificed nine rats were to compare the degree of re-epithelization and inflammation on 10th day while the remaining (9 animals) were sacrificed on day 20. Upon examination, significant difference was observed between the control and treatment groups in inflammation on 10th day and in re-epithelization on both 10th and 20th days ($p=0.05$). This led to the conclusion that wound contraction was significantly enhanced with irradiation of diode laser made incisions (Güngörmus and Akyol 2009).

Rodrigo et. al., 2009 undertook a research to evaluate, histologically, the systemic and repair process of three wounds created on the back of rats and directly or indirectly treated with red, infrared, or both lasers. They subjected four groups of rats to either red laser (AlGaInP), infrared laser (GaAlAs), both lasers, and no laser (control) with laser application fluency of 20J at 830nm or 685 nm. Three animals from each group were killed and subjected to evaluation (of skin reaction and wound healing), at 3, 5, and 7 days post operation.

Upon examination, they found more advanced healing in the wound located furthest from the point of laser application in both red and infrared laser groups. However, the first effective healing of a proximal wound was observed in the control group only on the 7th postoperative day. These led to their conclusion that the direct laser application to the wounds stimulated their healing, as well as healing of the wounds distant from the point of application, and that the combined application of red and infrared lasers 830nm

or 685 nm resulted in the most evident systemic effect on the repair of skin wounds produced in rats (Rodrigo et. al., 2009).

To ascertain the effects of pulsed continuous laser on wound healing in rats using pulsed and CW Erchonia 635nm LLLT, Alwatban and Zhang 2004 created elliptic wounds with scalpel on the backs of rats. After calculations, the percentage of relative wound healing was found to be better when 100Hz frequency was applied in pulsed mode. However, the best effect was obtained with CW. They thus concluded that pulsed continuous laser was not effective in increasing wound healing in rats compared with normal continuous laser (Alwatban and Zhang 2004).

In another study, Alwatban et. al., 2007 determined and compared the effects of various laser wavelengths and dosages on wound healing in diabetic rats. Full thickness Oval wounds were aseptically created on the skin of 51 Streptozotocin (70 mg/kg) induced diabetic and six non-diabetic male Sprague-Dawley rats. They used 532nm, 633nm, 810nm, and 980 nm diode lasers with incident doses of 5, 10, 20, and 30 J/cm^2 and treatment schedule of 3 sessions per week. They measured and plotted the area of wound of all rats on a slope chart, and computed the slope values (mm^2/day), percentage of relative wound healing, and the percentage of wound healing acceleration as evaluating parameters.

Analysis of results obtained showed that the Mean slope values were 6.0871 in non-diabetic control and 3.636 in diabetic control ($p < 0.005$). The percentages of wound healing acceleration were 15.23, 18.06, 19.54, and 20.39 with 532-nm laser, 33.53, 38.44, 32.05, and 16.45 with 633-nm laser, 15.72, 14.94, 9.62, and 7.76 with 810 nm laser, and 12.80, 16.32, 13.79, and 7.74 with 980 nm laser, using incident doses of 5, 10, 20, and 30 J/cm^2, respectively. Furthermore, they observed significant differences ($p < 0.001$) in the mean slope value of wound healing in diabetic rats between control and treatment groups in 532, 633, 810, and 980 nm lasers. They thus concluded that the healing of wounds in diabetic control was slower than in non-diabetic controls and that laser therapy using appropriate parameters can stimulate healing in diabetic rats. The optimum wavelength and incident dose being 633 nm, and10 J/cm^2 in for the study (Alwatban et. al., 2007).

In an in vivo study to ascertain the effect of LLLT on wound contraction, Demidova-Rice et. al,. 2007 used a filtered lamp to deliver a set of fluences of 635nm (±15nm). A

full thickness dorsal excisional wound in BALB/c mice was exposed to light 30 minutes post wounding with fluences of 1, 2, 10 and 50 J/cm^2 delivered at constant fluence rate of 100 mW/cm^2 and taking 10, 20, 100 and 500 seconds respectively.

Monochromatic coherent light from 632.8 nm He-Ne laser was used to deliver two fluences 1 and 2J/cm^2 (fluence rates of 2 and 1 mW/cm^2), respectively. Another monochromator coupled xenon arc lamp was used to produce 670 ± 15, 720 ± 15, and 820 ± 15nm light. Light delivery was with a ringed light guide, creating homogeneous light spot of 3 cm diameter (fluence of 1 J/cm^2 and fluence rates of 0.59, 0.79, and 0.86 mW/cm^2 for 670 ± 15, 720 ± 15, and 820 ± 15nm, respectively).

Upon measurement of wound area, they observed that the untreated wound tends to expand for 2-3 days post operation, but slight exposure to light immediately after wounding reduces or even stops the expansion leading to significant reduction in integrated time course of the wound size. They then hypothesized that there may have been transformation of dermal fibroblasts, in the edge of the wound, into myofibroblasts, thereby preventing wound expansion with their contractile smooth muscle actin fibers. They further observed a biphasic dose response curve for fluence of 635nm light with maximum effectiveness at 2 J/cm^2. They observed 820nm to be the best wavelength compared to 635, 670, and 720 nm. They also found no difference between non-coherent 635 ± 15nm light from a lamp and coherent 633nm light from a HeNe laser. Finally, their findings that low level laser therapy increased the number of α-smooth muscle actin (SMA) cells at the edge of the wound corroborated their hypothesis that low level laser therapy stimulated wound contraction in the tested mouse strains.

CHAPTER 4

MATERIALS AND METHODS

4.1 Experimental Animals

The experiment was conducted under an approved protocol of Institutional Animal Research and Care Ethics Committee at Bogazici University. Twelve (12) inbred male Wistar rats, randomly selected, 6–9 months old, weighing 200–220 g and skin thickness between 0.75 mm and 0.79 mm, from Boğaziçi University Vivarium, were used. They were housed in individually ventilated cages and maintained on a 12 hour light/12 hour dark cycle at 22±2°C temperature (and relative humidity of air at 50-70%) controlled vivarium. Food and water were available ad libitum.

4.2 Laser System

For this experiment, we used an infrared 808-nm wavelength GaAlAs diode laser (Teknofil Programmable Pulsed Laser Source TP-01 Power 3 W, Laser Class IV, 50 Hz, Power Input 230).

4.3 Experimental Design and Operations

The animals were divided into 4 groups of 3 animals each. Control group C (n=3), received no irradiation. The treatment groups were further subdivided into Inflammatory group (I) (n=3), Proliferation group (n=3) and Remodeling group (R) (n=3). The rats were intraperitoneally anesthetized with ketamine (100mg/kg) and Xylazine (10mg/kg)

(10%ketamidor, RichterPharma, AG, Wels, Austria) injection (1.65 ml/kg) both before wounding and during treatments. The hair at the site of application was shaved and a biopsy punch 5mm Acupunch Acuderm USA) was positioned perpendicular to the surface of the shaved skin, next to the midline, and close to the head of the animal. The punch was rotated to facilitate full skin penetration.

The wound diameter measured 5 mm, corresponding to that of the biopsy punch used. Soft tissue was carefully removed to obtain wound of about 0.7mm depth. The same procedures were followed to produce another wound about 6 cm from the first one.

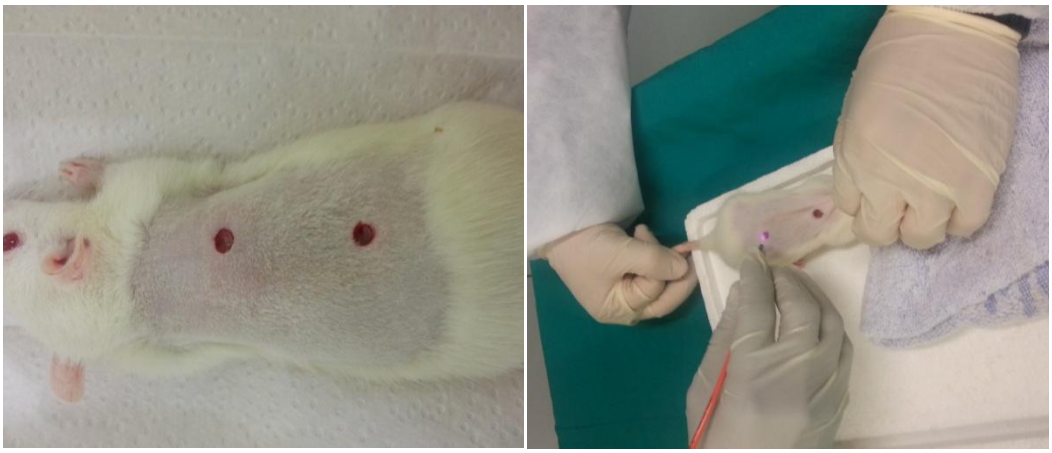

Figure 4.1 The wounds and the irradiation process.

We made topical application of antiseptic Poviiodex Scrub (Kim-Pa Ilaç Lab. Inc., Hadımköy, Istanbul, Turkey) on the shaved skin before each operation to minimize infection and desiccation. The wounds were left uncovered during the whole period of experiments; however, to inhibit any microbial reactions on the wound, Thiocilline (Abdi Ibrahim Ilaç Inc., Istanbul, Turkey) was applied on each wound. The diameters of the wounds were checked with digital calipers (Insize Digital Outside Caliper).

For the first group (inflammatory phase I), LLLT was started immediately after wounding and repeated 3 and 6 hours (3 sessions). During laser applications, 0.1 W of power was delivered to the tissue in 6 s, resulting in 5 J/cm^2 energy density (0.02 W/cm^2 power density) per session (15 J/cm^2 total dose). The irradiations were done spot by spot to cover the entire wound area with the tip of the optical fiber positioned approximately 1 cm above the wound during all laser applications, and the spot size of the laser beam was measured as 2 mm with a detection card (Thorlabs, Newton, NJ, USA), and laser power was checked with a power meter (Newport 1918-C, CA, USA). This dose was set after thorough review of related literature as reported above.

The second group (proliferative phase P), received LLLI of same dose at 24th, 26th and 30th hour (3 sessions) after wounding. The third group (remodeling phase R), received LLLI of same dose at 72nd, 76thth and 80th hour (3 sessions) after wounding.

Table 4.1 Laser Parameters.

Parameter	
Laser	GaAlAs semiconductor diode.
Wavelength (nm)	808
Wave Emission	Pulsed
Power Output (mW)	100
Power Density (W/cm^2)	0.02
Spot Size (cm^2)	0.2
Duty Cycle	015
Fluence ($J/cm^{2)}$	5
Duration of Irradiation (s)	6.67

We monitored recovery process up to 14 days and observed no adverse effects of wounding on animal health. We measured wound diameters with digital caliper (Insize Digital Outside Caliper) daily and wound areas calculated. Wound images were captured every other day using a digital camera.

The 3rd, 7th, and 14th, days of this period were set as control days, when skin samples were removed and submitted to histological processing as described below.

4.4 Histology

The histological samples were processed at Boğaziçi University Istanbul Turkey.

4.4.1 Tissue Processing

For histological analysis, 4 Wistar rats (one from each group: I1, P1, R1, and control) were anesthetized with a mixture of ketamine and xylazine solution and later sacrificed through cervical dislocation. 2 cm by 1 cm pieces of skin containing the wounded area were removed on 3rd, 7th and 14th postoperative days. The samples were placed in 10%

phosphate buffered saline (PBS)–formalin solution filled Falcon tubes until required for tissue processing.

The samples were inserted into pre-labeled plastic cassettes and loaded into the tissue processing machine (Leica TP 1020 with vacuum attachment) which took 18hrs to dehydrate, clear, permeate and fill the tissue samples with paraffin.

4.4.2 Paraffin Embedding

The samples were later transferred into metal cassettes and then embedded in paraffin blocks using the paraffin embedding machine (Leica EG 1150 H) and cooled using cold plate (Leica EG 1150C) and later stored at 4^{o}C for 24 hours before sectioning.

4.4.3 Tissue Sectioning

The samples were then sectioned to 10–12 μm-thick samples with a microtome (Leica RM 2255).

4.4.4 Hematoxylin & Eosin Staining

The slides were incubated (for 24hrs) and later stained with hematoxylin and eosin (H&E) for general histology. This procedure was carried out in a fume cupboard.

- Xylene -7minutes (dipped every minute).
- 100% Alcohol-2 minutes.
- 90% Alcohol- 2 minutes.
- Water- 3-4 minutes washing.
- Hematoxylin- 1.5 minutes.
- Water- 3-4 minutes.
- 90% Alcohol- 2 minutes.
- Eosin- 2 minutes.
- Water- 2 minutes.
- 90% Alcohol- 2 minutes.
- 90% Alcohol- 2 minutes.
- 100% Alcohol-2 minutes.
- Xylene-2.5 minutes.

After allowed to drip for a minute, a drop of Entellen was then applied on each tissue section and then the cover slip placed on top of the slide.

4.5 Histological and Morphometrical Analysis

The stained samples were used for descriptive and comparative microscopic analysis under light microscope (Nikon Eclipse 80i, Nikon Co., Japan). We used charge-coupled device (CCD) camera (OS-Fi1, Nikon Co.) at 4x magnification to capture images for analysis of wound healing and tissue repair process in the sections. Examination was done with imaging software (NIS Elements-D, Nikon Co. and Image J 1.47V).

4.6 Statistical Analysis

The data obtained were subjected to independent paired T test at 2 degrees of freedom and 0.05% p value using Microsoft Excel 2007. The percentage of wound healing acceleration and wound size reduction relative to control was calculated following Al-Watban and Zhang (1996).

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Results

During this research, all procedures and treatments were performed with no complications. The animals recovered well from anesthesia, indicating good health, physical activity and normal behavior. We observed no exudate in any of the wounds and gradual reduction in wound area progressively in the healing process of all three laser treated groups.

5.2 Morphometrical Analysis

5.2.1 Third Day Group

On the third day upon sacrifice and removal of wound area; the wound area in control group was not reduced (but rather increased) and wound is still visible on muscular layer with absence of neovascularization. However there was complete healing of muscular layer with visible neovascularization and formation of subcutaneous fatty layer in proliferative and inflammatory groups (Figure 5.7I3 & P3). A significant reduction in wound area was also observed in both groups (indicating wound contraction).

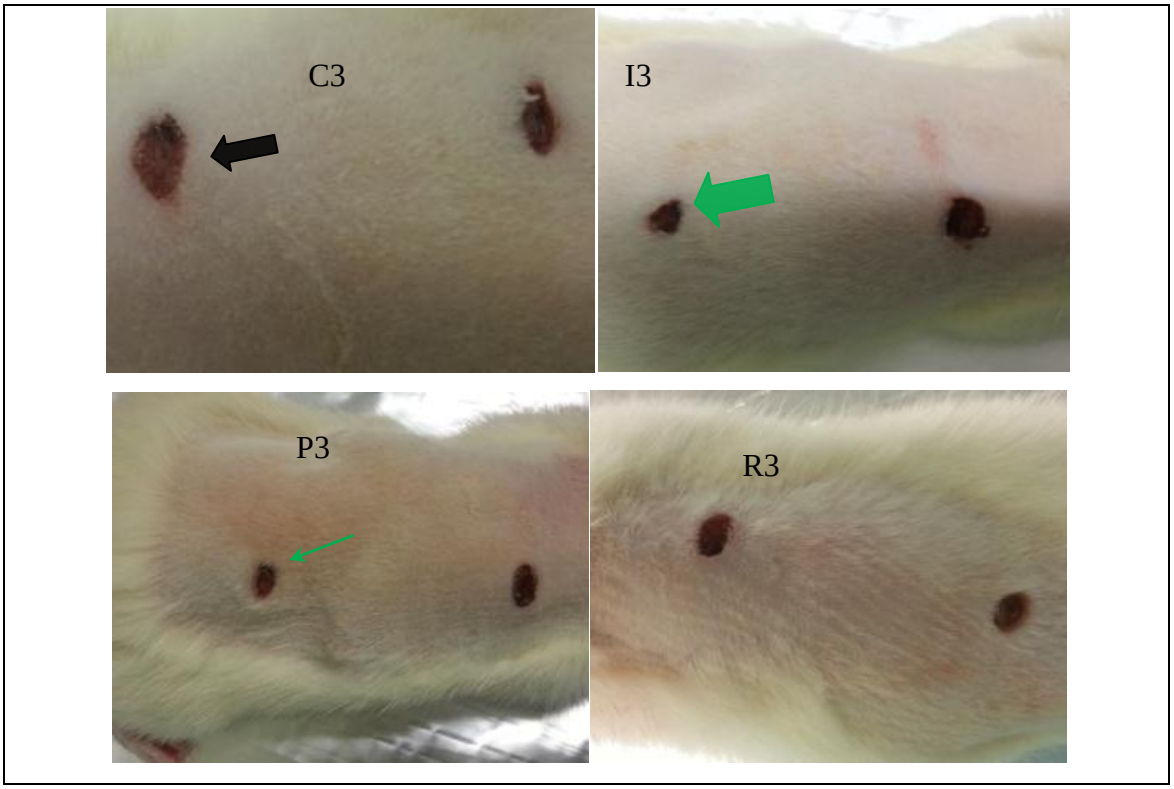

Figure 5.1 Wound Diameter of all groups on 3rd day post wounding.

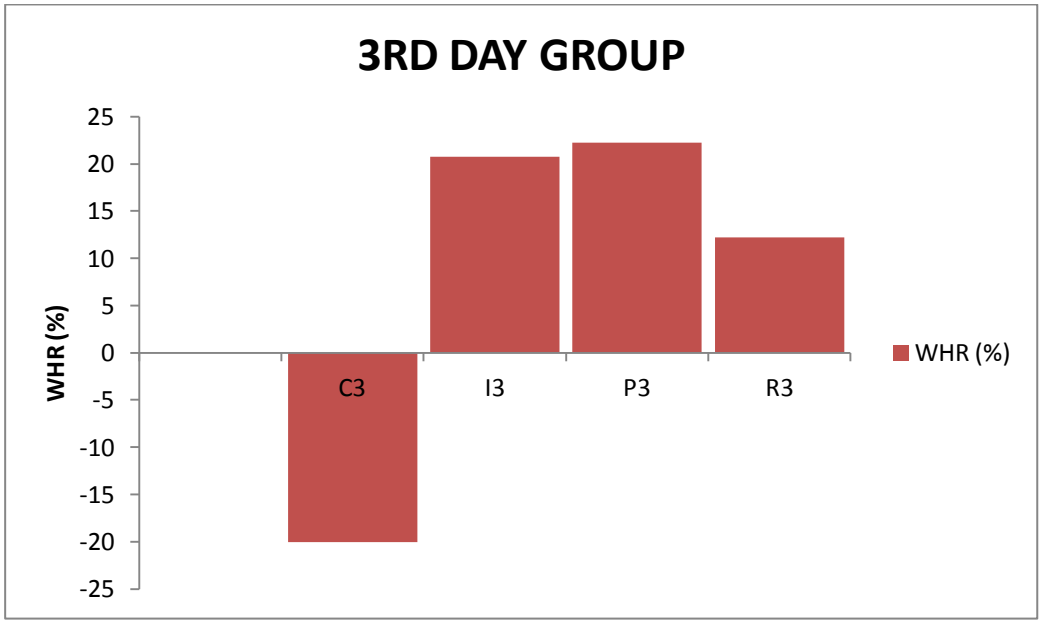

Figure 5.2 Graphical Representation of WHR (%) in 3rd Day Group.

5.2.2 Seventh Day Group

We observed slower healing processes in control group probably due to delayed epithelization by local inflammatory process. We also observed infiltration of inflammatory cells such as neutrophils, macrophages and lymphocytes in the dermis. This is also observed in the papillary dermis with additional fibroblastic infiltration (Figure 5.7C3). Analyses also showed part of the wounded skin still lacking epidermis with inflammatory process still present (Figure 5.7C7) and unorganized connective tissue, exhibiting few fibroblasts, capillaries, and collagen fibers.

However, in the deep dermis of laser treated groups, we observed connective tissue of young collagen with no hair follicles. Also, some connective tissue is transforming into skin muscle (Figure 5.7 I7, P7 & R7) with formed blood and lymphatic vessels increasing the blood circulation and lymphatic drainage. The predominant cells being macrophages, lymphocytes and fibroblasts.

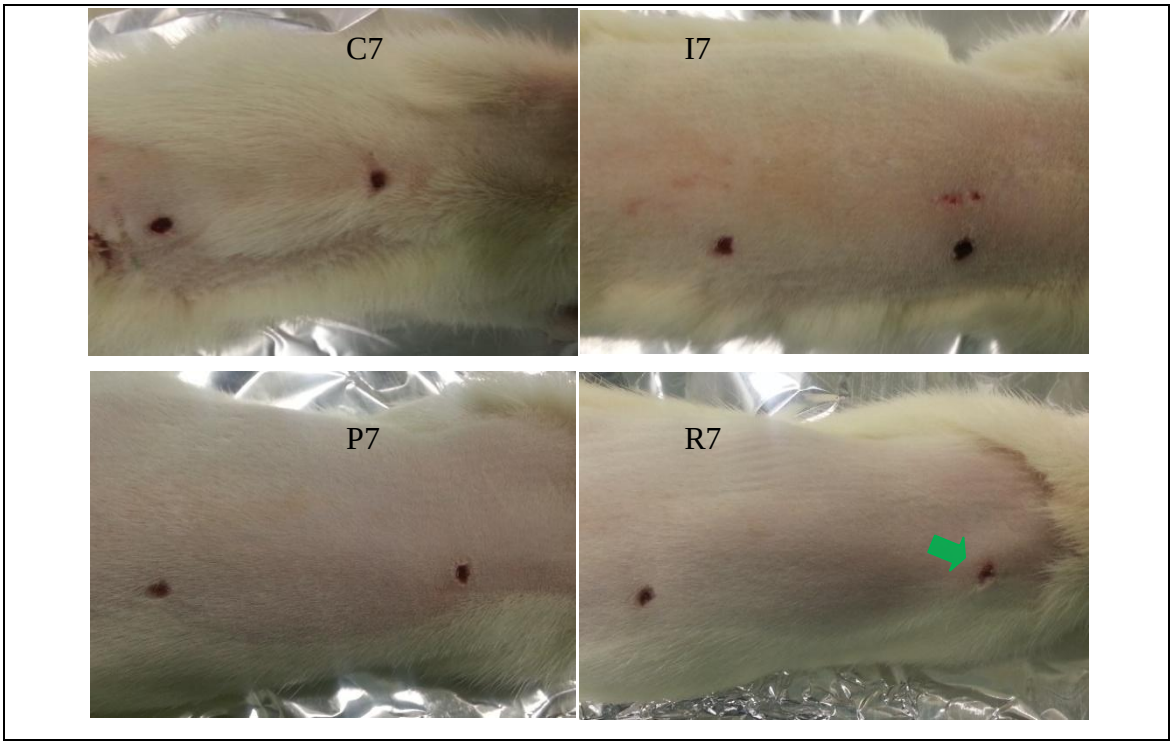

Figure 5.3 Wound Diameter of all groups 7th day post wounding.

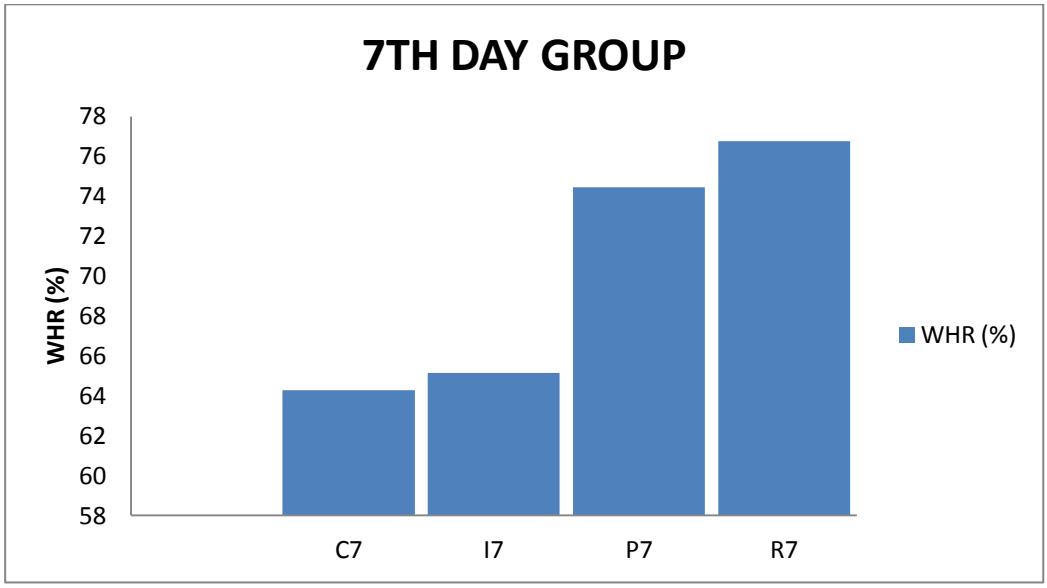

Figure 5.4 Graphical Representation of WHR (%) in 7th day group.

5.2.3 Fourteenth Day Group:

On the 14th day, in the control group, next to the wound edge, a discrete epithelial proliferation was observed, and the presence of tissue all over its extension exhibiting a broad ulceration material over granulation tissue (Fig 5.7 C14).

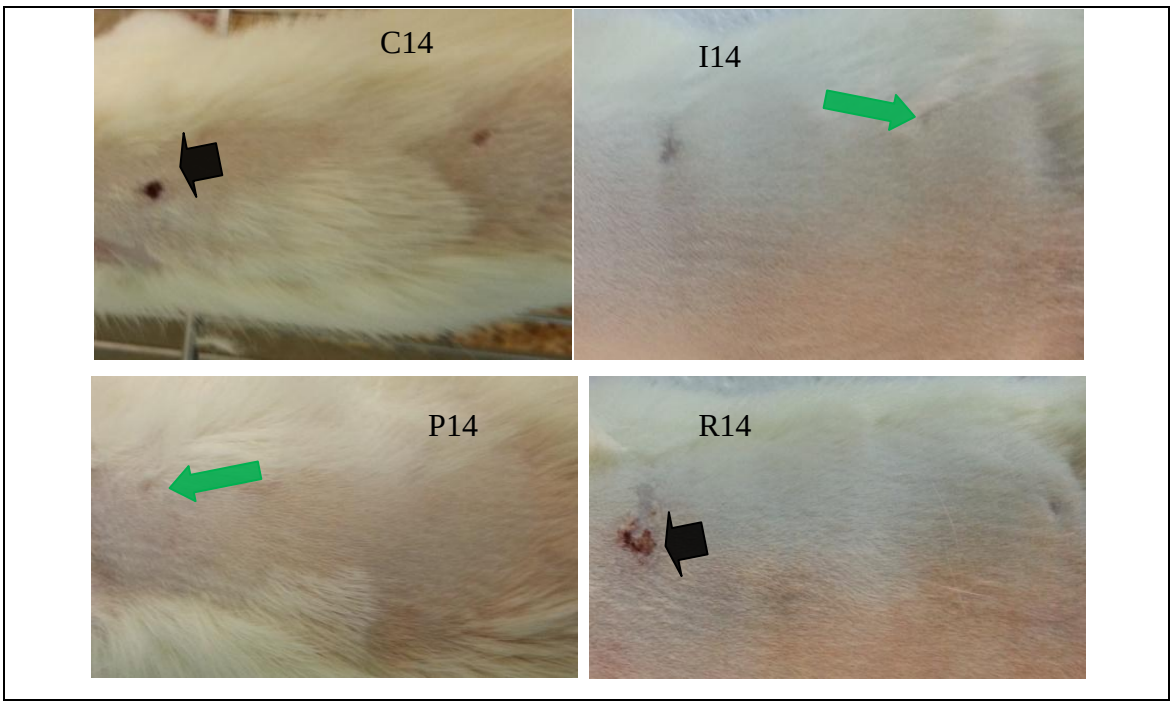

Figure 5.5 Wound Diameter of all groups (%) 14th day post wounding.

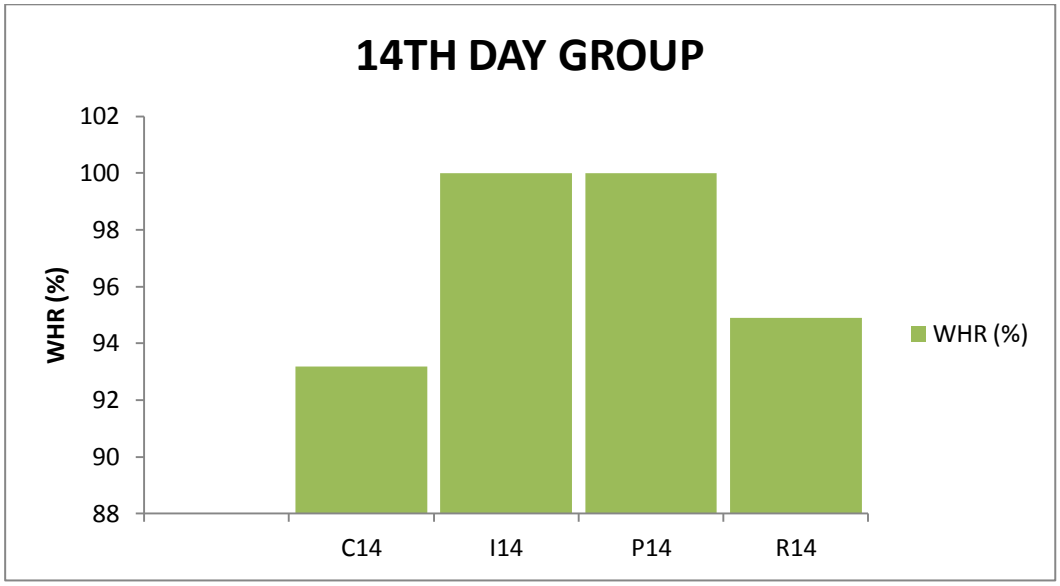

Figure 5.6 Graphical Representation of WHR (%) in 14th Day Group.

5.3 Histological Analysis

In the analysis of the histological results, both dermal and epidermal areas were evaluated. Histological examinations also revealed differences between control and treated groups right from third day post wounding, when laser irradiated animals present good wound edge approximation, with partial epithelialization (Figure 5.7 I3, P3 and R3), unlike the control group where inflammation persists (Figure 5.7 C3).

We observed some differences between irradiated and control groups regarding the characteristics of the wounded dermis. Re-formed capillaries, fibroblasts and small collagen fibers were observed in the laser treated wounds. For groups I7 and P7, epidermis was almost completely formed. However, samples from R7 presented less organized connective tissue than P7. A variety of inflammatory cells such as lymphocytes and macrophages were observed in I7, but not in P7. The samples from P7 also exhibited higher fibroblast cells content and more newly formed capillaries than R7.

Additionally, irradiated animals, especially the proliferative group, presents intact healed epidermis covering well-developed granulation tissue (Figure 5.7 I14, P14 and R14), with connective tissue rich in collagen fibers with a parallel orientation with regard to the surface of the wound indicating a more organized healing process. For the inflammatory and proliferative groups, the healing process is over in 14 days (Figure 5.7 I14, P14).

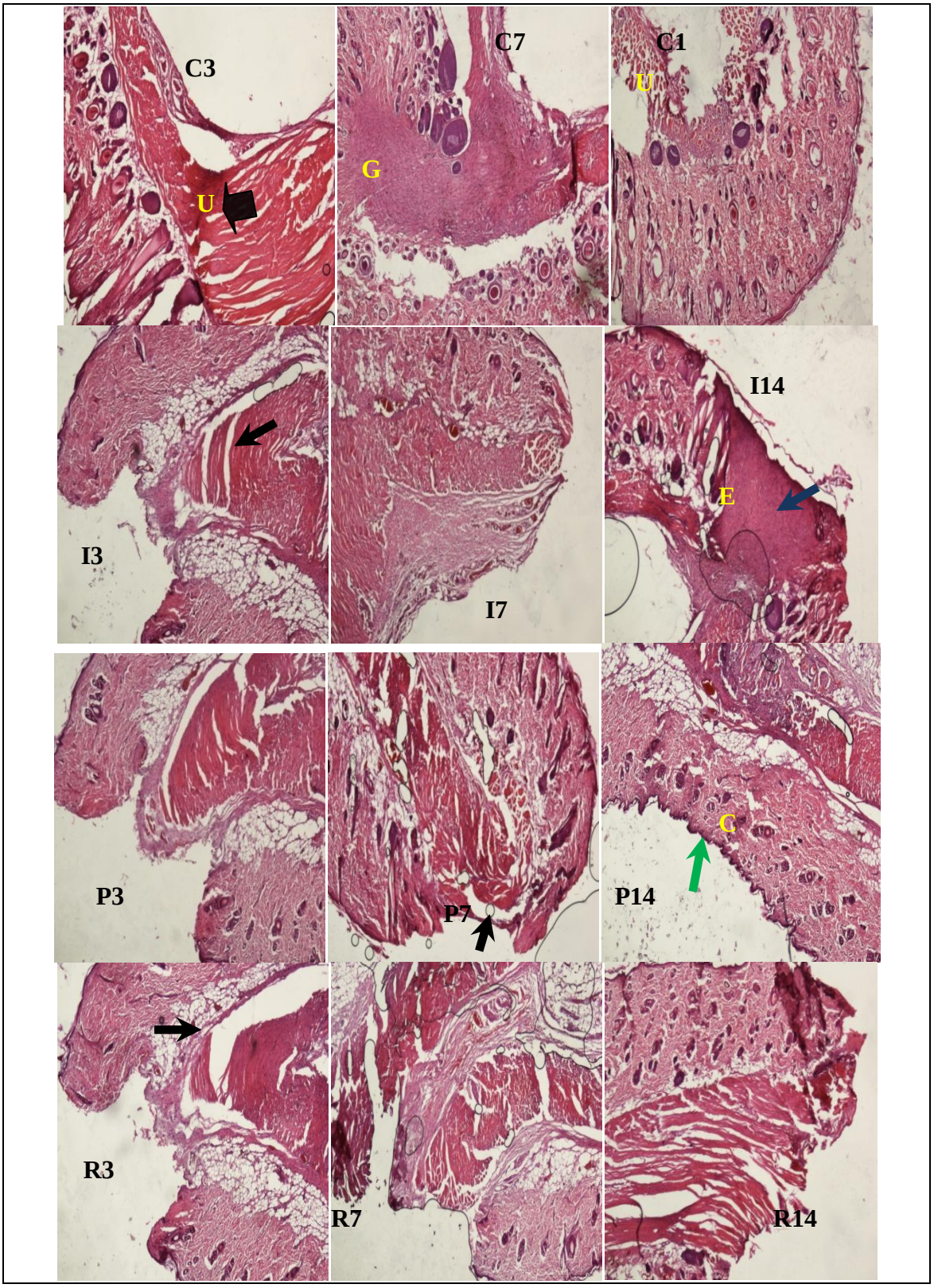

Figure 5.7 Photomicrographs of the H&E stained samples after 3, 7 and 14 days of healing showing the effect of LLLT on wound repair process. U= Ulceration, G= Granulation Tissue, E= Epithelialization, C= Collagen

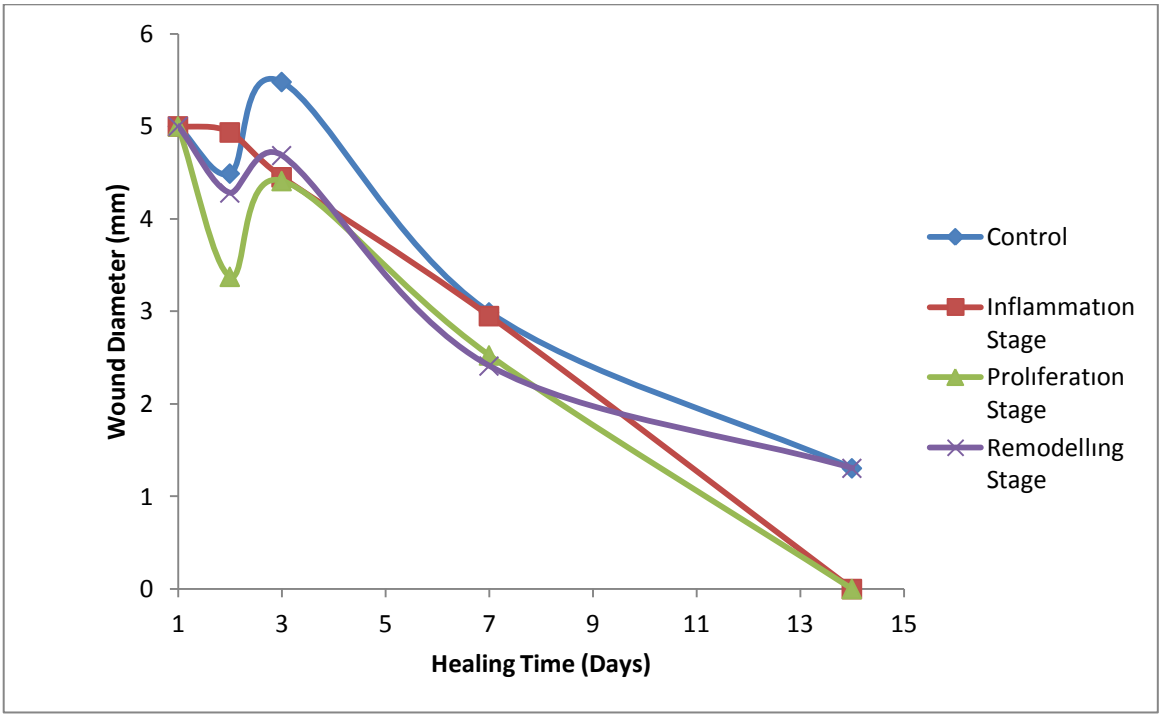

Figure 5.8 Variation of wound diameter with healing time.

Table 5.1 Mean ± Standard Deviation for each Group.

MEAN ± STANDARD DEVIATION PER GROUP				
Group	**C**	**I**	**P**	**R**
Mean±SD	45.62±48.29	61.97±32.36	65.56±35.50	61.27±35.50

Statistical analysis of Mean WHR (%) (Figure 5.9) for inflammatory, proliferative and remodeling groups were found to differ significantly ($p>0.05$) from the control group with lower mean value from the control group than the test groups. However, no statistically significant difference ($p>0.05$) was found between proliferative and inflammatory groups with the former having higher mean value. Upon comparing proliferative and remodeling groups, significant difference ($p>0.05$) was found with higher mean value from proliferative group. Additionally, no significant difference was found between remodeling and inflammatory groups with the former having slightly higher mean value.

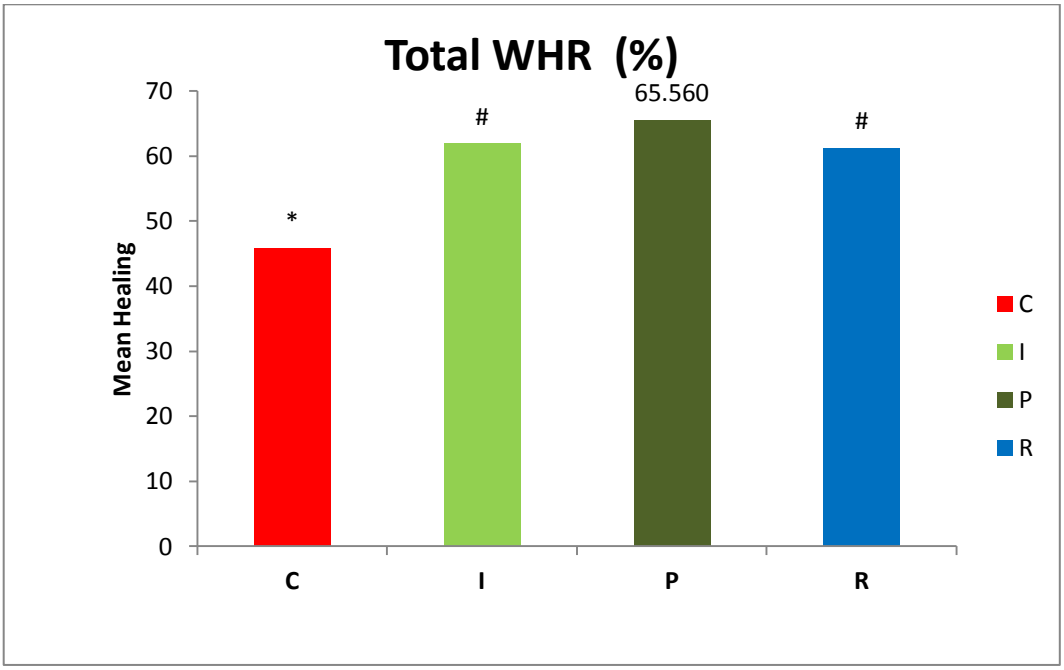

Figure 5.9 Mean Healing for each group across 14 days healing period. ***** Denotes statistically significant difference when compared to other groups. # Denotes no Statistically significant difference when compared.

5.4 Discussion

In this study, we observed that the effects of 15 J/cm^2 LLLT on punch wounds are more evident in the early stages of the healing process. However, we couldn't find much significant effect of LLLI applied at later stages of wound healing when compared with control (in accordance with Ezzati and bayat 2009). Thus, apparently, LLLT was more effective when applied 24hrs after wounding. Our result is in accordance with that of Rezende et. al. 2007 and that of Alwatban and Zhang 2004 who observed some improvements after animal treatment particularly in the earlier phases of wound healing.

The biostimulative effect of pulsed LLLI in the current study is demonstrated by the significant increase in the rate of wound contraction in irradiated groups when compared with the control 3, 7, and 14 days after wounding, Our observations of enhanced wound healing rates in the test groups were corroborated by the histological results obtained as evident in the increased granulation tissue, proliferating fibroblasts as well as collagen deposition and orientation.

On the other hand the control group was probably delayed in a chronic inflammatory stage as evidenced by ulceration and lack of granulation tissue. Moreover, it was

observed that LLLT significantly reduced the intensity of the inflammatory infiltrate present in the wounds submitted to treatment. In addition, neovascularization, fibroblast proliferation and reduced inflammatory infiltrate in treated wound submitted to laser therapy were observed. This is in accordance with the works of Roca et. al., 2006 who observed low-intensity laser treated wounds showed better tissue repair process, with higher rate of wound retraction and greater epithelial migration when compared to the wounds in the control group.

A trend of wound diameter reduction followed by abrupt increase was observed in all groups except inflammatory group (figure 5.8). This might be explained by the resultant vasodilation resulting after LLLI (to ensure increased blood flow to the area and thus more oxygen and nutrients) as explained by Stadelman et. al., 1998. For the non increase in wound diameter of inflammatory group, the explanation is as thus; immediately after wounding, there is immediate vasoconstriction wich might have shadowed and overcomed the vasodilatatory effect of our laser irradiation.

For the control group such observation might be due to morbidity, persistent macrophageal action or as a result of the individual physiology of the rat. However, in the opinion of Montandon et. al., 1977, the decreased contraction observed in round wounds is often a secondary phenomenon due to the lack of extensibility or adherence to the surrounding skin.

Additionally, this observation is in consistence with the findings of Demidova-rice et. al., 2007 who upon measurement of wound area, observed that in their model, the untreated wound tends to expand for 2-3 days after it was made, but even a brief exposure to light soon after wounding, reduced or stops its expansion, thereby reducing the integrated time course of the wound size. They then hypothesized that fibroblasts in the edge of the wounded dermis might have been transformed into myofibroblasts, and the contractile nature of these cells with their smooth muscle actin fibers (increased at the wound edge) prevents the wound expanding.

The healing process in the control group was slow throughout the healing days. On the 7th postoperative day, the wounds were not healed as primary healing seems delayed. The wound was not fully epithelialized even on the 14th postoperative day as shown by the histological examination of tissue obtained.

We observed better healing in irradiated wounds than in the control group; Statistical analysis showed a significant difference between the groups treated with laser than the control group (Figure 5.9). Researches into mechanisms involved have shown that LLLT can stimulate many of the cell types whose interactions repair the dermis both in vitro and in vivo. This stimulation has been shown to result mast cells and macrophages to release growth factors and other substances, whereas the proliferation of fibroblasts, endothelial cells, and keratinocytes maintained during adverse conditions can also be stimulated. Matic et. al., 2003 also reported that the development of granulation tissue is mainly controlled by growth factors released from macrophages.

Additionally, the facilitation of wound contraction observed is as explained by Lawrence 1998 and in accordance with the works of Hussein et. al., 2011 who reported that laser irradiation transforms fibroblasts into myofibroblasts which are directly involved in granulation tissue contraction and thus wound contraction.

The results of the present study, Ezzati and Bayat, and that of Matic et. al., 2009 who observed significant acceleration in rate of wound closure of a rectangular acute wound of all skin layers in their study, confirmed the positive effect of pulsed LLLT on skin wound using a GaAlAs 808 nm.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

From the morphometrical, histological and statistical results obtained, it can clearly be inferred that LLLT has best effect when first applied about 24hrs post wounding (late inflammatory, early proliferative stage) as evident in the increased granulation tissue, fibroblasts and collagen deposition and orientation observed, leading to faster wound contracture and thus healing.

6.2 Recommendations

It is very important that correct protocols are developed for laser therapy and that they include the use of appropriate wavelength, dose, potency density, irradiation time, as well as frequency and number of sessions as these parameters may have an influence on treatment outcome and avoid controversies and empirical conclusions.

The many variable dosage and delivery parameters may in part explain the lack of reproducibility of results obtained. There is need for standardized ideal dose for stimulating tissue healing. This will go a long way in enabling the establishment of criteria on the true benefits of laser therapy in circumstances requiring stimulation of healing, minimizing healing time and possible complications during the progression of healing. In addition, regulation of thermal effects is imperative to ensure that observations are not due to heating phenomenon.

Finally, simultaneous application of LLLT and topical treatment should be tested.

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APPENDIX A

Laser Dosimetry Calculation

t= D*A / P* (1+d)

Time of treatment= t

Dosage Wanted= D= 5J/cm^2

Area Treated= A= 0.2cm^2 (0.2mm^2)

Power = P= 0.1W (100mW)

Depth of 1 to 4cm= d

= 5 J/cm^2*0.2cm^2 / 0.1W* (1+0.5cm) = 5s

APPENDIX B

Calculation of Wound Healing Rate

Wound Healing Rate (%) = $(1 - (A_n/A_1))* 100\%$

A_n Area of wound on day n

A_1 Area of wound on day 1

(Alwatban and Zhang 1996)

APPENDIX C

Experimental groups and Laser Application protocol.

Groups	**Control**	**Treatment**		
Subgroups	C	I	P	R
Number n	3	3	3	3
Irradiation Time Hours(Post wounding)	-	0,3 and 6	24,26 and 30	72, 74 and 76
Power mW	-	100	100	100
Power Density W/cm^2	-	0.02	0.02	0.02
Energy Density J/cm^2	-	5	5	5
Laser Mode	-	Pulsed	Pulsed	Pulsed

APPENDIX D

Calculations for Mean ± Standard Deviation for groups (14 days)

STAT FOR MEAN ± STANDARD DEVIATION					
Control Group					
	WHR of C	**C-Mean**	**Y=(C-Mean)2**	**Variance ∑Y/n**	**SD √∑Y/n**
Day 3	-20.600	-66.220	4385.088	2331.746	48.288
Day 7	64.280	18.660	348.196		
Day 14	93.180	47.560	2261.954		
n	3.000				
∑	136.860		6995.238		
Mean	45.620				

STAT FOR MEAN ± STANDARD DEVIATION					
Inflammation Group					
	WHR of I	**I-Mean**	**Y=(I-Mean)2**	**Variance ∑Y/n**	**SD √∑Y/n**
Day 3	20.770	-41.197	1697.165	1051.235	32.423
Day 7	65.130	3.163	10.007		
Day 14	100.000	38.033	1446.534		
n	3.000				
∑	185.900		3153.706		
Mean	61.967				

APPENDIX D Contd.

STAT FOR MEAN ± STANDARD DEVIATION					
Proliferation Group					
	WHR of P	**P-Mean**	**Y=(P-Mean)2**	**Variance ∑Y/n**	**SD √∑Y/n**
Day 3	22.240	-43.320	1876.622	1047.197	32.360
Day 7	74.440	8.880	78.854		
Day 14	100.000	34.440	1186.114		
n	3.000				
∑	196.680		3141.590		
Mean	65.560				

STAT FOR MEAN ± STANDARD DEVIATION					
Remodeling Group					
	WHR of R	**R-Mean**	**Y=(R-Mean)2**	**Variance ∑Y/n**	**SD √∑Y/n**
Day 3	12.180	-49.093	2410.155	1259.921	35.495
Day 7	76.750	15.477	239.527		
Day 14	94.890	33.617	1130.080		
n	3.000				
∑	183.820		3779.763		
Mean	61.273				

APPENDIX E

Wound Healing Rate Calculation

Initial Diameter (mm)	**5**				
Initial Area (mm^2)	**19.635**				
3RD DAY GROUP	**DIAMETER**		**Mean**	**Area (mm^2)**	**WHR (%)**
	F	**B**			
C3	5.568	5.389	5.479	23.573	-20.06
I3	4.805	4.096	4.451	15.556	20.77
P3	4.071	4.747	4.409	15.268	22.24
R3	5.333	4.038	4.686	17.243	12.18

Initial Diameter (mm)	**5**				
Initial Area (mm^2)	**19.635**				
7TH DAY GROUP	**DIAMETER**		**Mean**	**Area (mm^2)**	**WHR (%)**
	F	**B**			
C7	3.022	2.955	2.989	7.014	64.28
I7	3.202	2.703	2.953	6.847	65.13
P7	2.362	2.694	2.528	5.019	74.44
R7	2.621	2.201	2.411	4.565	76.75

APPENDIX E
Contd.

Initial Diameter (mm)	**5**				
Initial Area (mm^2)	**19.635**				
14TH DAY GROUP	**DIAMETER**		**Mean**	**Area (mm^2)**	**WHR (%)**
	F	**B**			
C14	2.612	0	1.306	1.340	93.18
I14	0	0	0.000	0.000	100.00
P14	0	0	0.000	0.000	100.00
R14	2.261	0	1.131	1.004	94.89

APPENDIX F

Wound Diameters

Healing (days)	WOUND DIAMETER (mm)					
	Control			Inflammation		
	F	b	mean	F	b	mean
1	5.000	5.000	5.000	5.000	5.000	5.000
2	4.780	4.200	4.490	4.640	5.230	4.935
3	5.570	5.390	5.480	4.810	4.090	4.450
7	3.020	2.960	2.990	3.200	2.700	2.950
14	2.610	0.000	1.305	0.000	0.000	0.000

Healing (days)	WOUND DIAMETER (mm)					
	Proliferation			Remodeling		
	F	b	mean	F	b	mean
1	5.000	5.000	5.000	5.000	5.000	5.000
2	3.610	3.140	3.375	4.190	4.370	4.280
3	4.070	4.750	4.410	5.330	4.040	4.685
7	2.360	2.690	2.525	2.620	2.200	2.410
14	0.000	0.000	0.000	2.610	0.000	1.305

APPENDIX G

Wound Healing (14 Days)

WHR Total Healing Days				
GROUPS				
	C	**I**	**P**	**R**
Day 3	-20.060	20.770	22.240	12.180
Day 7	64.280	65.130	74.440	76.750
Day 14	93.180	100.000	100.000	94.890
∑	137.400	185.900	196.680	183.820
Mean	45.800	61.967	65.560	61.273

APPENDIX H

T-test

WHR (%)									
	I	**C**	**d**	**d^2**	**Mean ∂**	**Variance S^2d**	**SD**	**T2 Value**	**DF**
Day 3	20.770	-20.060	40.830	1667.089	16.167	465.120	268.537	2.960	0.05
Day 7	65.130	64.280	0.850	0.722					
Day 14	100.000	93.180	6.820	46.512					
Count	3.000								
∑	185.900	137.400	48.500	1714.324					
Mean	61.967	45.800							

WHR (%)									
	P	**C**	**d**	**d^2**	**Mean ∂**	**Variance S^2d**	**SD**	**T2 Value**	**DF**
Day 3	22.240	-20.060	42.300	1789.290	19.760	383.828	221.603	3.982	0.05
Day 7	74.440	64.280	10.160	103.226					
Day 14	100.000	93.180	6.820	46.512					
Count	3.000								
∑	196.680	137.400	59.280	1939.028					
Mean	65.560	45.800							

APPENDIX H Contd.

WHR (%)									
	R	**C**	**d**	**d^2**	**Mean ∂**	**Variance S^2d**	**SD**	**T2 Value**	**DF**
Day 3	12.180	-20.060	32.240	1039.418	15.473	239.785	138.440	3.945	0.05
Day 7	76.750	64.280	12.470	155.501					
Day 14	94.890	93.180	1.710	2.924					
Count	3.000								
∑	183.820	137.400	46.420	1197.843					
Mean	61.273	45.800							

WHR (%)									
	P	**I**	**d**	**d^2**	**Mean ∂**	**Variance S^2d**	**SD**	**T2 Value**	**DF**
Day 3	22.240	20.770	1.470	2.161	3.593	25.050	14.463	2.835	0.05
Day 7	74.440	65.130	9.310	86.676					
Day 14	100.000	100.000	0.000	0.000					
Count	3.000								
∑	196.680	185.900	10.780	88.837					
Mean	65.560	61.967							

WHR (%)									
	P	**R**	**d**	**d^2**	**Mean ∂**	**Variance S^2d**	**SD**	**T2 Value**	**DF**
Day 3	22.240	12.180	10.060	101.204	4.287	38.763	22.380	2.718	0.05
Day 7	74.440	76.750	-2.310	5.336					
Day 14	100.000	94.890	5.110	26.112					
Count	3.000								
∑	196.680	183.820	12.860	132.652					
Mean	65.560	61.273							

WHR (%)									
	I	**R**	**d**	**d^2**	**Mean ∂**	**Variance S^2d**	**SD**	**T2 Value**	**DF**
Day 3	20.770	12.180	8.590	73.788	0.692	116.724	67.390	0.253	0.05
Day 7	65.130	76.750	-11.620	135.024					
Day 14	100.000	94.894	5.106	26.071					
Count	3.000								
∑	185.900	183.824	2.076	234.884					
Mean	61.967	61.275							

CURRICULUM VITAE

Name Surname: **Musbahu Muhammad Sani**

Place and Date of Birth: **Kano 1-November-1989**

Address: No 119 Tudun Nufawa Quarters Kano, Nigeria.

E-Mail: almusby@yahoo.com

B.Sc.: Biochemistry (Bayero University Kano)

M.Sc.: In View