THE EFFECT OF INFRA-RED LASER THERAPY ON FIBROBLAST ACTIVITY IN CELL CULTURE.

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Submitted in partial fulfilment of the requirements for the degree

Master of Medical Science

In the Department of Physiology

Faculty of Medicine

University of Natal

1997.

ABSTRACT

Fibroblasts play an important role in wound healing and constitute a major part of granulation tissue. Human fibroblasts were cultured *in vitro* and irradiated with different energy densities of 830nm continuous output infrared laser generated by a Gallium Aluminium Arsenide Endolaser 476. The fibroblasts were irradiated on three consecutive days at energy densities of 0.2, 0.4, 0.6, 0.8, 1.5, 2.3, 3, 4, and 5J/cm^2 delivered at an average power output of 30mW, and at a constant distance of 1cm from the fibroblasts. Fibroblast proliferation was assessed on the fourth day using the MTT assay. There was a significant increase in fibroblast proliferation at laser treatment energy densities of 0.4J/cm^2 and 5J/cm^2. In a new model of wound healing, fibroblasts were grown on silicone discs and the discs "wounded" by creating a 1cm diameter defect in the centre of the disc. The discs were irradiated for 3 consecutive days at energy densities of 1J/cm^2 and 4J/cm^2. An unlasered disc served as a control. Fibroblast proliferation was enhanced at both energy densities in the wounded models. Difficulties associated with *in vivo* and *in vitro* studies of the effect of laser treatment are discussed.

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged.

The research was carried out in the Department of Physiology, University of Natal Medical School under the supervision of Mr AA Chuturgoon.

SUSAN MARS

1997

ACKNOWLEDGEMENTS

Mr AA Chuturgoon for his supervision and assistance throughout this project.

Ms D Pillay for teaching me cell culture techniques and assisting with the biochemical assays.

Ms E Gouws for statistical advice.

Professor M Mars for his support and guidance.

Professor JV Robbs and the Wound Healing Research Group for funding this project.

Churchill Livingstone for permission to reproduce certain illustrations.

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CHAPTER 1.

INTRODUCTION

In the clinical situation low intensity laser therapy (L.I.L.T.) is an accepted, efficient, noninvasive and painless method of treating oedema, inflammation and pain, and of increasing circulation and promoting wound healing (Basford, 1989).

Healing is a natural process occurring continuously in the body. Low intensity laser therapy is thought to provide a biostimulatory effect, enhancing this natural process (Brom, 1994). Mester *et al* (1985) demonstrated that laser promoted wound healing by increasing collagen formation, vasodilatation and DNA synthesis. Although favourable responses to wound healing have been reported following L.I.L.T. (Mester and Jaszsagi-Nagy, 1973; Dyson and Young, 1985; Dyson and Young, 1986; Enwemeka *et al*, 1990), there is, however, uncertainty regarding optimal dosage regimens. The clinician is faced with the problem that there is no generally agreed or scientifically validated dose for laser therapy in humans (Kitchen and Partridge, 1991) and the dose related efficacy of L.I.L.T. remains elusive (Enwemeka, 1988). This is due in part to inadequate reporting of dosage in terms of laser power, area of application, duration and frequency of treatment using different methods, lasers and models (Baxter, 1994). Further research in the form of thoroughly documented, controlled studies is necessary (Miller and Dyson, 1996).

The aim of this study is to establish the effectiveness of infra-red Gallium Aluminium Arsenide (Ga-Al-As) laser therapy (830nm) on stimulating human fibroblast activity in cell culture, and to determine the optimal dose required to stimulate fibroblast activity *in vitro*. This study further aims to define the energy dose range that provides maximum fibroblast proliferation in a new *in vitro* model of wound healing.

CHAPTER 2.

LASER

This chapter deals with the concept of laser, its properties and investigates the factors influencing the interaction of laser and the target tissue. The physiological effects of laser are summarised.

2.1 WHAT IS LASER?

Laser is an acronym for Light Amplification by Stimulated Emission of Radiation. The laser produces a unique form of electromagnetic energy in the visible or infrared regions of the electromagnetic spectrum. Electrons of an active medium are stimulated (elevated from the ground state). They emit radiation and return to their normal energy state. The emitted photons collide with other excited photons causing additional emission of energy. This is termed the "knock-on effect".

The laser is named after its active medium which may be a solid semiconductor as in the GaAlAs laser, or gas as in the helium-neon laser.

2.2 THE ELECTROMAGNETIC SPECTRUM

The electromagnetic spectrum is made up of different radiations comprising alternating electrical and magnetic fields which fluctuate in synchrony and perpendicular to the

direction of propagation. The distinguishing factors are the frequency and wavelength of the radiations. The relationship between frequency and wavelength is expressed by the equation

$$\lambda = c/f$$

where λ = the wavelength (metres)

c = speed (metres per second)

f = frequency (hertz per second)

L.I.L.T. utilises the 600 - 1 000 nm wavelength range which includes visible red radiation at the lower end of the visible range and invisible near infra-red radiation.

2.3 PRODUCTION OF LASER

Laser light is created by an energy source and the photons of light emitted are then amplified by reflection between two mirrors. One mirror is totally reflective and the other is partially reflective. The energy source is the "heart" of the laser and located in the probe. The emitted energy in the form of a stream of photons interacts selectively with elements in biological structures. These biological interactions are wavelength dependent.

The production of laser by the semiconductor GaAlAs laser used in this study is different.

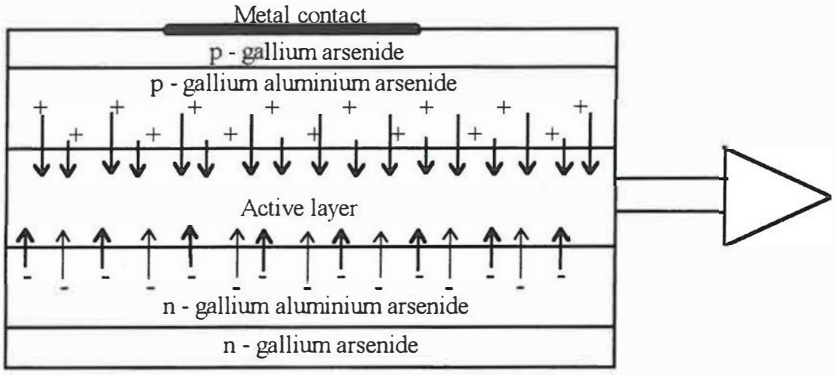

Figure 1. Semiconductor laser : gross structure.

An external electrical source causes positively charged holes to be injected down from the p-type gallium aluminium arsenide layer into the active layer of gallium arsenide. Simultaneously electrons are driven up from the n-type layer of gallium aluminium arsenide into the active layer. As the holes and electrons interact photons of light are produced. The exact wavelength of the light is determined by the amount of aluminium in the gallium aluminium arsenide layer (Figure 1). The photons which strike the highly polished ends of the semiconductor at right angles reflect forward and backwards causing photon resonance in the active layer and consequent laser light emission (Baxter, 1994).

2.4 PHYSICAL PROPERTIES OF LASER ENERGY

Laser devices produce intense beams of light and differ from ordinary light (Figure 2) by being monochromatic, directional and coherent. The emitted energy may be continuous or pulsed. Where pulsing units are used, average radiant power output can

vary significantly with altered pulse repetition rate. It is therefore important to monitor the power output (Baxter, 1994).

2.4.1 Monochromaticity: refers to the emission of a single wavelength of light, and is considered to be the most important attribute of laser (Figure 3).

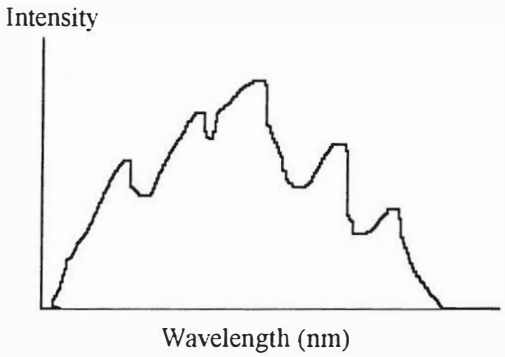

Figure 2. Emission of a non laser light source eg lightbulb.

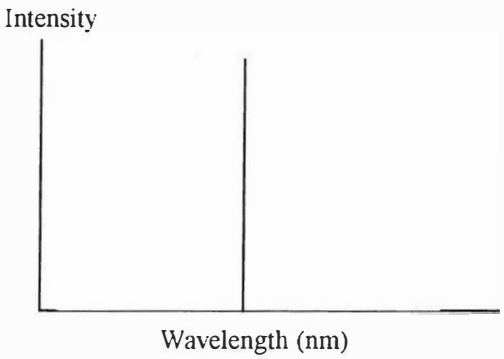

Figure 3. Emission of a laser light source.

2.4.2 Collimation and divergence. Non-laser light radiates uniformly in all directions, while laser output is highly collimated. Most laser light is emitted in parallel with 3 - 10 degrees of divergence reported for an average diode-based system (Baxter,

1994) (Figure 4). In the clinical situation, the laser probe should be in contact with the target tissue. Power output is measured at the point of emission and the intensity decreases proportionately as the distance from the target tissue increases, the inverse square law.

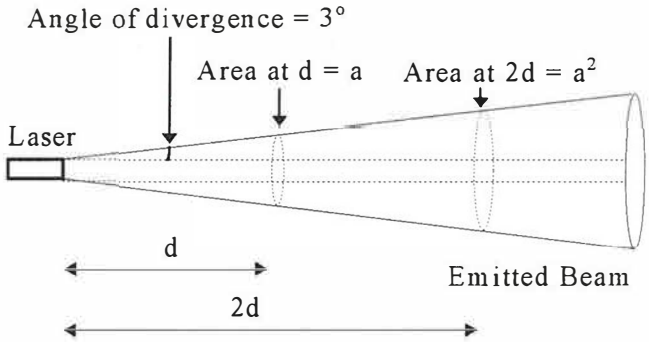

Figure 4. Divergence. Doubling the distance between the source and the target tissue squares the area of irradiation. The intensity of radiation is therefore proportionately decreased.

2.4.3 Coherence refers to the inherent 'synchronicity' of the emitted laser beam. The photons are in phase or "step", both temporally and spatially (Figures 5 and 6).

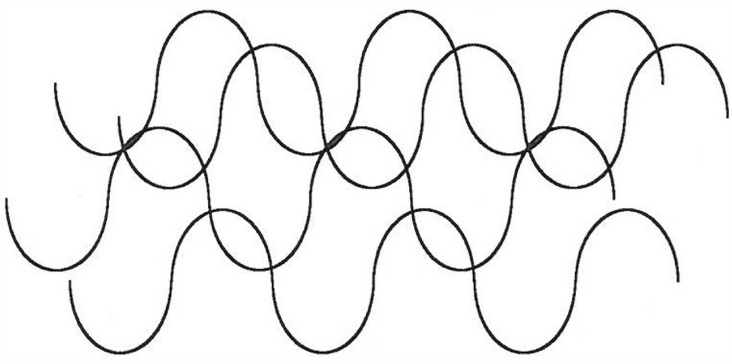

Figure 5. The light irradiation is asynchronous in non coherent (non - laser) light.

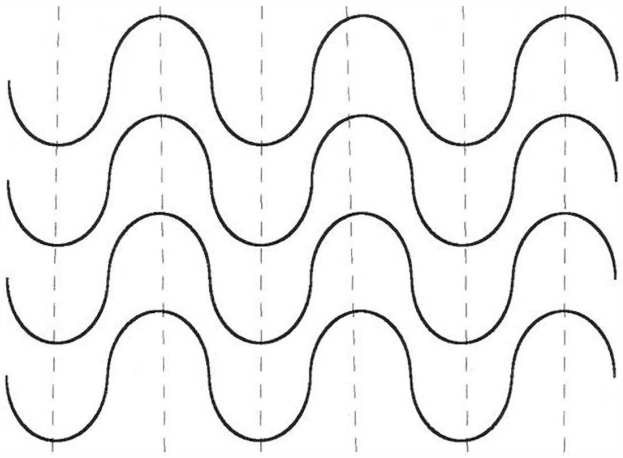

Figure 6. Coherent light irradiation from a laser source.

Karu (1987) and Young, *et al* (1989) questioned the importance of coherence as a major factor in photobiological responses, as non-coherent lasers also produce a photostimulative effect on macrophages and fibroblasts. Laser emitting super luminous diodes (SLDs), which lack the attribute of coherence, may provide an adequate photostimulative effect. They are a cheaper source of laser light.

The output of the laser and the effective dosage delivered by L.I.L.T. is best described in terms of irradiance or power density, where irradiance is the quotient of incident power of the light and the area irradiated.

$$\text{Irradiance/Power density (mW/cm}^2\text{)} = \frac{\text{Incident (mW) power}}{\text{Area of irradiation (cm}^2\text{)}}$$

2.5 LASER EQUIPMENT

Commercially available lasers range from the He-Ne laser machine, which is large, cumbersome, expensive and hazardous to the eyes because of its highly collimated beam, to semi-conductor diode lasers such as GaAs, GaAlAs which make use of diode-based systems. These are cheaper, portable, easier to use and offer the option of a continuous or pulsed laser beam.

2.6 APPLICATION

International safety standards (Baxter, 1994) have been set for the four classes of laser. For L.I.L.T. these pertain mainly to ocular hazard, requiring both the recipient and the therapist to wear protective goggles. It is also necessary to provide adequate verbal warnings of the dangers of laser. Observation of safety rules and care in avoiding treatment of conditions in which laser therapy is contraindicated is mandatory.

Contraindications to laser therapy include.

1. Treatment of the eye
2. Treatment of neoplasms
3. Treatment over the pregnant uterus / foetus
4. Treatment of haemorrhaging wounds
5. Treatment of acutely infected open wounds
6. Treatment over the gonads, thyroid gland or over the cardiac region
7. Patients undergoing photosensitive treatment
8. Epileptics

Laser can be applied on a local point, or may be scanned over a grid system of individual points in a large area such as a burn. Alternatively a cluster probe made of several diodes emitting various wavelengths simultaneously, may be used. The power density will vary with the individual diode output and geometry.

The power output may be monitored by the unit itself once energy density or time is chosen as is the case with the Endo-laser 476. Energy density is the product of power density and time, and is expressed in Joules/cm^2.

$$\text{Energy} = \frac{\text{Power (mW)} \times \text{Time (sec)}}{\text{Area } (cm^2)}$$

The effects of laser are dependent not only on wavelength but also the nature of the tissue to which it is applied. The laser beam may be transmitted, reflected, scattered or absorbed. With the probe held at right angles to the skin, about 5% of the incident light is reflected on striking the epidermis.

Scattering refers to a directional change of light propagation due to the complex geometry of the tissue involved. Most scattering occurs in the dermis (Parrish, 1982), with minimal scattering in the epidermis. Ninety percent of scattering that occurs at therapeutic wavelengths (660 - 1 300nm) is in a forward direction, as opposed to back or random, isotropic scattering. Scattering depends on the relationship between the wavelength of the laser beam and the size of the biomolecules, for example collagen,

encountered. The longer wavelengths (820nm) penetrate the dermis better than shorter wavelengths (632nm).

Absorption is essential for a biostimulatory effect. The amount of absorption in the different skin layers is affected by the skin's thickness, blood flow, water content, amino acid content and the presence of chromophores. Chromophores are light absorbing pigments such as melanin, bilirubin, haemoglobin and β carotene. Light energy is converted to other types of energy during absorption. Scattering and absorption determine the depth of penetration of the beam.

Absorption appears to depend on the type of tissue and the wavelength of the laser. Various models and theories exist as to the amount of light actually absorbed by tissue. Beer has put forward an absorption model while Mie has proposed a theory based on scattering. The radiative transfer theory provides an attractive unidirectional solution, while the Monte Carlo model offers a three dimensional solution (Baxter, 1994). Detailed discussion of these models is beyond the scope of this study.

The rate of absorption of energy at any point within the tissue is termed the "fluence rate" which is the product of the fluence (ϕ) and the tissue's absorption coefficient (a), where fluence is the power of the laser divided by the depth at which the absorption is occurring.

$$\text{Fluence rate } (W/cm^3) = a\ (cm^{-1}) \times \phi\ (W.cm^{-2})$$

The depth of penetration of the beam is dependent on wavelength, scattering and absorption and is taken as the depth at which the intensity of the beam is 36% of the original incident intensity. The beam emitted from a laser probe is however not uniform or homogenous but Gaussian in distribution. The beam imprint has a bell-shaped profile with the intensity at the edges of the beam being 12.9% of the intensity of the central strength of the beam (Baxter, 1994).

In practice, irradiance or power density of the treatment unit, is the total incident power divided by the spot size of its beam on the tissue surface.

$$\text{Energy density } (J/cm^2) = \frac{\text{Power (W) x time (sec)}}{\text{area } (cm^2)}$$

2.7 HISTORICAL BACKGROUND

Planck and Einstein first developed laser concept at the turn of the century. Subsequently Maxwell speculated about electromagnetic radiation waves and Planck proposed the quantal theory of photons of energy. In 1960 Dr Theodore Maimam finally produced a ruby crystal laser radiation device. This was followed by helium-neon and carbon dioxide lasers. These lasers found rapid application in medicine and surgery.

Opthalmologists soon used the surgical laser to secure detached retinas. Short pulsed Laser therapy was used next in the field of oncology but this was short-lived as the

explosive debris resulting from laser therapy was deemed a possible source of metastases (Minton *et al*, 1965). More recently, laser has been used intravascularly to disobliterate vessels stenosed by atheromatous plaques.

In the 1970's, CO_2 laser was found to be more suitable for surgical use. The lasers used surgically are all hard or high powered lasers. For wound healing, low powered or soft L.I.L.T. lasers are used. Unlike high power laser, L.I.L.T. does not rely on photothermal or ablative interactions of laser and tissue (Baxter, 1994).

Low intensity laser therapy research was pioneered by Plog in Canada and Mester in Budapest. Mester *et al*, (1985) were surprised by the photobiostimulation effect and recovery of chronic wounds *in vivo*. Plog (1980) studied the effect of laser on acupuncture points versus invasive needling. The results were encouraging and much of the subsequent research was done in Eastern Europe, China and the Soviet Union.

2.8 APPLICATION OF LASER TECHNOLOGY

1. Commercial uses include laser light shows, lecture pointers and supermarket scanning devices.
2. Surgical lasers cause thermal changes in the target tissue e.g. surgical incision or destruction of tumours using for example the Carbon dioxide laser (wavelength 1060nm)
3. Low energy lasers stimulate tissue but cause no thermal changes in the target tissue. Examples are the He-Ne Laser and infrared.

Low intensity laser therapy is currently used in the treatment of musculoskeletal and nerve injuries, enhancement of wound healing, for local analgesia and to reduce inflammation.

2.9 PHYSIOLOGICAL EFFECTS OF L.I.L.T.

Infra Red (IR) laser light ± 632nm appears to be the most "energising" frequency of laser at a cellular level (De Bie and Steenbruggen, 1989). Its skin penetration depth on average is 0.5 - 1cm.

2.9.1 BIOSTIMULATION

Karu (1987) found that IR laser (620nm) stimulated bacterial cell culture growth rate (*Escherichia coli*), DNA and RNA synthesis rates, enzyme activity and cAMP levels. It was postulated that the respiratory chain was being stimulated, activating ATP turnover, increasing H^+, and ultimately triggering increased cell proliferation.

The stimulating effects of light appear to occur in "sluggish" cell culture or in circumstances of decreased activity such as in trophic ulcers and indolent wounds, where low tissue oxygen concentration and pH inhibit cell growth. Light irradiation appears to upregulate cellular metabolism and proliferation. Conversely, where maximum regeneration is occurring naturally, laser did not appear to enhance the process (Lam, 1986).

The positive biostimulatory effects of L.I.L.T are summarised as follows:

- Laser has a non-thermal, catalysing effect accelerating cell metabolism (Mester *et al*, 1985).
- Fibrocytes and macrophages enhanced activity (Dyson and Young, 1985; Young *et al*, 1989; Bolton *et al*, 1991)
- The tissues are more efficiently cleared of oedema, vasodilatory agents disappear; inflammation is reduced (De Cuyper, 1984).
- Effects include:
    - cellular proliferation, motility and phagocytosis (Tocco *et al*, 1985)
    - increased collagen, (Mester and Jaszsagi-Nagy, 1973; Enwemeka, 1989) ATP and protein synthesis (Bosatra *et al*, 1984)
    - increased prostaglandin synthesis (Basford, 1989; Young *et al* 1989)
    - increased intracellular matrix, neurotransmitter release, transmembrane potential, oxyhaemoglobin dissociation and cell granule release (Basford, 1989)
    - increased cell proliferation particularly fibroblasts (Boulton, 1990)
    - increased collagen synthesis and tensile strength (Lyons *et al*, 1987)
    - increased myofibroblast proliferation (Dyson, 1986; Pourreau-Schneider *et al*, 1990)
    - increased changes in cell mitochondrial function (Tocco *et al*, 1985;, Karu, 1988)
    - increased monocyte stimulation (Young *et al*, 1989)
    - accelerated fracture healing (Trelles *et al*, 1987)

- increased mineralization of the fracture callus (Chen and Zhou, 1989)

The only negative effect on cell proliferation was a reduction in lymphocytes which Inoue *et al* (1989) suggest is consistent with the observed effect of reduced inflammation.

Recently the Guys Hospital Tissue Repair Unit reported that L.I.L.T. had no beneficial healing effect on experimental wounds *in vitro* (Evans *et al*, 1995). Most work has been done *in vitro* and this is difficult to compare with *in vivo* studies.

2.9.2 LASER - TISSUE INTERACTION

Low intensity laser therapy does not cause any appreciable temperature changes and is thus classified as an athermic modality. It is best applied in contact with the target tissue so that maximal absorption and minimal scattering and divergence occurs.

Light absorption occurs at a molecular level through excitation of the electron bonds within the biomolecules, exciting atoms to higher modes of oscillation relative to each other and through rotation of the whole or part of the biomolecule by the electromagnetic field created by the laser light (Baxter, 1984). The organic molecules responsible for light absorption are divided into two groups, the amino acids and nucleic acid bases, and chromophores.

Scattering occurs in the tissues due to the complex geometry of biomolecules.

CHAPTER 3

LITERATURE REVIEW

Three areas of research are reviewed for laser photobiomodulation of wound healing.

1. In vitro studies of cell culture
2. Animal studies
3. Human studies

A brief review of wound healing is included.

3.1 PATHOPHYSIOLOGY OF WOUND HEALING

This complex physiological process commences at the time of injury. The immune and circulatory systems are stimulated. Cell movement, division and several chemical and cellular responses occur. The three overlapping phases of healing are the inflammatory phase, followed by the proliferative phase and matrix remodelling (Clark 1985,1988) (Figure 7).

3.1.1 INFLAMMATORY PHASE

The acute inflammatory phase lasts 1-3 days. A cascade of events occurs after the injury as a result of blood and lymph vessel damage, platelet aggregation, degranulation and blood coagulation. The initial vasoconstriction controls the haemorrhage. Vasodilatation and erythema follow. Leukocytes, especially neutrophils,

appear at the injured site. Oedema develops secondary to increased capillary endothelial permeability. The damaged cells release histamine and other active mediators such as prostaglandins and serotonin which enhance the process.

Neutrophils marginate and leave the vascular compartment by diapedesis. Macrophages also appear. They phagocytose debris and release biologically active substances such as vasoactive mediators, and chemotactic and growth factors. The chemotactic substance fibronectin, is necessary to recruit fibroblasts to the injured area (Tsukamoto *et al*, 1981) and to facilitate collagen deposition.

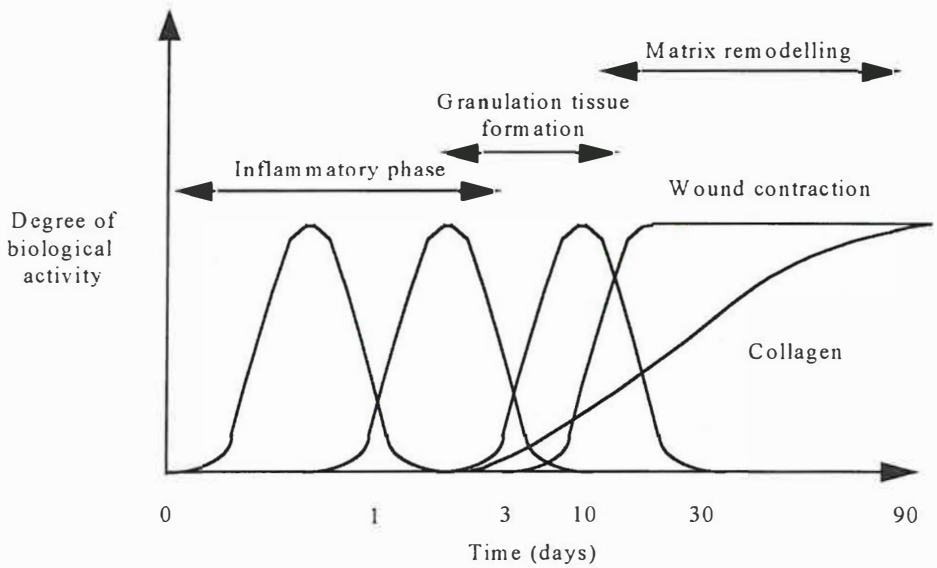

Figure 7. Triphasic model of wound healing.

3.1.2 PROLIFERATIVE PHASE

The stage of re-epithelialisation and contraction occurs over the next 10 - 14 days. Regeneration of the epidermis, neo-angiogenesis and fibroplasia leading to collagen

synthesis occurs during this phase. Fibroblasts are microscopically visible from about day two onwards. Macrophages also encourage fibroplasia and angio-genesis by releasing growth factors at the wound site (Silver, 1984).

Re-epithelialisation begins within 24 hours after injury. Fibroblasts proliferate and during granulation fibroblasts may be transformed to actin rich myofibroblasts which have the ability to contract and move over each other thus contributing to wound contraction (Clark, 1988; Gabbiani *et al*, 1972). This process known as interssusceptive growth results in a smaller wound (Montanden *et al*, 1977). Myofibroblasts synthesise collagen after 5 days and this increases the tensile strength of the healing tissue.

3.1.3 MATRIX REMODELLING

During this stage collagen fibres that have been randomly laid down are reorganised. Collagen alignment increases the mechanical strength of the wound to stretch. This phase may continue for several months after the wound has healed (Enwemeka, 1988).

Occasionally where disturbance of the balance between collagen formation and lysis occurs, a keloid or hypertrophic scar may occur. Keloids are due to an over-deposition of collagen in both injured and peripheral wound tissue, while a hypertrophic scar results from an over-production of collagen in injured tissue only. These imbalances have recently been linked to abnormally raised cytokine concentrations in keloid tissue (Baxter, 1994).

3.2 CELLULAR RESEARCH

Early laser studies were confined to *in vitro* studies because little was known about the side effects of laser irradiation (Enwemeka, 1988). More studies have therefore been performed in this area than in any other. The majority of studies have shown beneficial effects, and most of the work has been performed using the helium neon (He-Ne) 632.5nm laser as super luminous diode lasers only became available after 1981.

Wound healing studies have focused on several types of cells including fibroblasts, lymphocytes, monocytes, macrophages, epithelial and endothelial cells. The wide diversity of experimental protocols and parameters such as cell line, dose, waveform, treatment time, penetration distance, treatment area and treatment frequency make comparison of these studies difficult.

3.2.1 FIBROBLASTS

Much of the *in vitro* research work has focused on fibroblasts which are primarily responsible for wound healing. The major effect of laser irradiation on fibroblasts is cell proliferation (Abergel *et al*, 1984 a; Boulton and Marshall, 1986).

Boulton and Marshall (1986) reported a significant increase in human embryonic foreskin fibroblasts following He-Ne laser irradiation. In a second study on adult human skin fibroblasts, proliferation was equally positive, using a He-Ne laser with

50% pulsed output for 15 minutes over 3 days. No further dosimetry details were available.

Pourreau-Schneider *et al* (1990) found that normal human gingival fibroblasts irradiated *in vitro* with He-Ne laser were massively transformed to myofibroblasts as early as 24 hours after treatment, when compared to control fibroblasts. In a similar study utilising gingival tissue biopsies taken after wisdom tooth extraction, laser irradiated gum tissue showed myofibroblast presence 48 hours after treatment. This was not seen in untreated control tissue. This is taken as evidence that He-Ne laser has a primary biologic effect on connective tissue, by generation of myofibroblasts from fibroblasts.

Not all results have been positive. Colver and Priestley (1989) studied seven components of wound healing *in vitro* after irradiation with He-Ne laser (633nm). A treatment dose of 5mW was applied three times a day over a 3 week period. The surface area irradiated was not stated. No significant effects were recorded. It is possible that the dose may have been inadequate. Bolton *et al* (1990 and 1991) felt that maximal stimulatory energy densities are 2.4 -7.2 Joules/cm^2.

Alternative mechanisms are that lasers (He-Ne and Ga ALAs) stimulate fibroblastic production of collagen. Abergel *et al* (1987) reported that low-energy GaAlAs lasers enhanced collagen gene expression both in skin fibroblast cultures *in vitro* and in animal models of wound healing *in vivo*.

Bosatra *et al* (1984) found that laser irradiation facilitated ulcer healing in the clinical situation as well as stimulating fibroblast growth *in vitro*. The He-Ne laser with a maximum output of 1.56mW increased collagen production. Although collagen production was increased after irradiation, periodic collagen fibril formation was not observed suggesting that complete 'restitution and integrum' of the dermal matrix does not occur. This conclusion may not be valid as collagen needs to be stress loaded to mature correctly (Woo *et al*, 1987).

Abergel *et al* (1984a) in their studies utilising keloid fibroblast cultures found that the neodynium -yttrium - aluminium - garnet (Nd : YAG) laser of wave-length 1 060nm and an energy density of 60J/cm^2 selectively suppressed collagen production in these cells. Based on the *in vitro* study, 8 patients with keloids or hypertrophic scars were successfully treated with Nd:YAG laser, and at 3 year follow-up the scars remained flat and soft.

The same group, Abergel *et al* (1984b) found that the Nd:YAG laser selectively suppressed collagen production both in fibroblast culture and normal skin *in vivo*. In contrast, the two low-energy lasers, He-Ne and Ga-As stimulated collagen production in human skin fibroblast cultures. Thus bio-inhibition of cell proliferation by the YAG laser, as observed by Abergel *et al* (1984b) and Dyson and Young (1985) is potentially of therapeutic value in the management of keloids and Dupuytren's contracture.

Zugaro *et al* (1991) applied low dosages of infrared laser to *in vitro* fibroblast cultures and after 12 days of irradiation found a great increase in cellular density. They

concluded that cellular mitosis correlated directly to the treatment. No details of dosage were given. In 1992 the same group investigated the effect of a different exposure time on fibroblast cultures and were unable to demonstrate any difference between treated and untreated fibroblasts. Again the exact treatment parameters were not detailed.

Lubart *et al* (1992) studied the effects of visible and near infrared (360, 632, 780 nm) lasers on fibroblasts in cell culture at various energy doses. Mitosis counts were taken and the authors suggested that proliferation was due to stimulation of cell respiration by intercellular endogenous porphyrins or cytochromes. The energy doses were not reported.

Using the He-Ne (632.8nm) laser, Van Breugel and Bar (1992) concluded that laser exposure time and power density determines the effect of the laser. Dependent on the exposure time and power density, the laser can either stimulate or inhibit the human fibroblasts *in vitro*. Cells were irradiated at various energy doses for three consecutive days for periods of 0.5 - 10 minutes, and at power outputs of 0.55 - 5.98 mW. Power outputs below 2.91 mW enhanced cell proliferation while 5.98mW had no effect. The surface area irradiated was not stated. Irradiation times of 0.5 to 2 minutes were most stimulatory. Of interest was the observation that cellular proliferation was associated with a reduction in Collagen type I production.

3.2.2 OTHER CELLS: LYMPHOCYTES, MACROPHAGES, ENDOTHELIAL CELLS AND MITOCHONDRIA

3.2.2.1 LYMPHOCYTES

Lymphocytes are responsible among other functions, for the release of mediators of the immune system. Laser irradiation studies of lymphocytes show decreased proliferation of these cells after treatment. This trend was shown by Mester *et al* (1978) using Argon and He-Ne lasers, Ohta *et al* (1987) (904nm laser) and Inoue *et al* (1989), (780 nm laser).

Inoue (1989) and Mester (1978) found clinically, that laser irradiation may suppress some of the undesirable immunoreactions. This was proposed as a possible explanation of improved healing of chronic wounds following laser treatment.

3.2.2.2 MACROPHAGES

Macrophages are multi-functional cells which remove debris, regulate the immune and inflammatory response and release factors to stimulate fibroblast proliferation and wound healing. In all the experiments on macrophages, the technique used was to laser the macrophages in solution and then to add the macrophage solution to a fibroblast culture well or plate.

Young *et al* (1989) investigated the effect of light on calcium uptake by macrophages using varying energy densities, pulse frequencies and wavelengths. They found the greatest response to be between frequencies 16 - 36.48 Hz, at energy densities of 4-8 J/cm^2 and at wavelengths of 660, 820 and 870nm.

When lasering macrophages using a super luminous diode laser pulsing at 120 mW/cm^2, and at energy densities of 2.4 - 9.6 J/cm^2, Bolton (1991) found 660nm to be the most stimulatory wavelength. Maximum fibroblast proliferation occurred at 7.2 J/cm^2, but dropped off at 9.6 J/cm^2. This suggests that the high energy densities may alter the cell metabolism or stimulate release of fibroblast inhibitory factors.

Young *et al* (1989) irradiated macrophages with wavelengths 660nm (non coherent), 820nm (coherent), 870nm (non coherent) and 880nm (non coherent) laser to stimulate release of mediators of wound repair from the macrophages. The effect of these mediators as reflected by fibroblast proliferation was assessed for 5 days and compared with controls. The 660nm laser caused maximal stimulation, whereas 880nm had an inhibitory effect at energy densities of 2-4 J/cm^2. The results suggest that the 880nm (non coherent) laser may be useful for inhibition of tissue growth in for example hypertrophic scars, as either fibroblast proliferation or factor release is inhibited at this wavelength. Conversely the results suggest that 660, 820 and 870 nm wavelengths may be used to stimulate fibroblast activity.

Bolton *et al* (1991) used a laser of 820nm wavelength, at a power density of 400 or $800mW/cm^2$ and an energy density 2.4 or 7.2 J/cm^2. Similarities in results were

obtained with 2.4 J/cm^2 and 800mW/cm^2 and 7.2 J/cm^2 and 400 mW/cm^2, whereas 7.2 J/cm^2 and 800mW/cm^2 and 2.4J/cm^2 and 400mW/cm^2 differed little from the controls. These results are interesting because previous experiments found energy densities between 2 - 4 J/cm^2 to be optimal. This study indicates that response is also power density dependent, and suggests that high power density and high energy density together reduce fibroblast proliferation. Possible proposed explanations of this are that various light receptors may be damaged, or that cytotoxic products may be formed in the cells thereby affecting the cell's respiratory metabolism. Alternatively the higher power density, coupled with the higher energy density may stimulate the release of fibroblast inhibitory growth factors.

3.2.2.3 ENDOTHELIAL CELLS

Ghali and Dyson (1992) irradiated bovine aortic endothelial cells with 660nm and 820nm laser. Energy densities were 1,2,4,8 J/cm^2, at a frequency 18 - 24 Hz. Cells were irradiated once and assessed over a 5 day period. 8 J/cm^2 resulted in greatest proliferation with 660 nm laser, while the 820nm laser at 1 J/cm^2 inhibited cell turnover.

3.2.2.4 MITOCHONDRIA

Bosatra *et al* (1984), and Tocco *et al* (1985) and found that laser stimulated fibroblast proliferation and also observed increased signs of protein metabolism and an increase in number and size of mitochondria in irradiated cells.

Karu (1988) irradiated isolated mitochondria and suggested that laser stimulated certain components of the respiratory chain which act as photo-acceptors. This resulted in activation of the electron transport chain in turn enhancing ATP synthesis. Hydrogen ion concentrations within the mitochondria are affected and sodium, potassium and calcium flux between mitochondria and cytoplasm is altered. Cell metabolic changes are ultimately involved in initiation of DNA synthesis.

Rajarathan *et al* (1994) irradiated macrophages with a pulsed laser at frequencies of 2 - 1 000 Hz, at a power output of 50mW and at a constant energy density of 7.2 J/cm^2. The range of pulsing frequencies used was 2.28, 18.24, 292.30 and 1 000 Hz. Fibroblast proliferation was assessed over 5 days. All frequencies except 1 000 Hz increased fibroblast proliferation when compared with controls. The cells treated with 1 000 Hz proliferated less than the controls. Results at 72 hours showed no difference between groups but at 120 hours post plating there were large increases in the lasered versus the sham irradiation group. They postulated that there is a frequency window at 18.24 Hz when laser wavelength is 820 nm, at which maximum proliferation or stimulation of wound healing occurs.

The concept of a frequency window at 18.24Hz was supported by a study by Ghali and Dyson (1992), who showed similar results on endothelial cell proliferation. They showed that the greatest proliferation occurs after a single irradiation of 660nm wavelength, energy density 8 J/cm^2 and frequency 18.24Hz and is maximal after 5 days.

3.3 ANIMAL STUDIES

Most animal studies have been performed on small, loose skinned rodents (rats, mice, and guinea pigs), for reasons of ease of handling, procurement and cost. Wounds in rodents heal largely by wound contraction which is different to the process of epithelialisation in humans. This must be borne in mind when comparing results between the two groups. The pig is more suitable for human skin comparison, however its skin is thicker. Laser penetration is therefore altered and the wound may only be superficially irradiated. (Hunter *et al*, 1984; Basford, 1986).

The difficulty encountered when reviewing animal studies of wound healing is the diversity, and lack of detail in the experimental reports.

The pioneering animal wound healing studies were done in the 1960's and 70's by Mester using a ruby laser. The best healing results were obtained at energy densities of 1 - 4 J/cm^2 and maximal collagen synthesis occurred at 4 J/cm^2 (Mester and Jaszsagi-Nagy, 1973). These initial studies tried to correlate the laser's biostimulatory effects with the biochemical changes occurring during healing.

Collagen content and tensile strength are often used as criteria of wound healing. Kana *et al* (1981) used the He-Ne laser and also found that $4J/cm^2$ produced significant increases in collagen content as well as wound closure in rats. The Argon laser was shown to have no effect on wound closure but improved collagen content (Jongsma *et al*, 1983; Mc Caughan *et al* 1985).

Hunter *et al* (1984) studied the effects of He-Ne laser on partial thickness wound healing in pigs. The energy density used was 0.96 J/cm^2 as a daily dose for three weeks. Results of the treated wounds were no better than the controls. Based on other reports the dose may have been too low to produce a beneficial effect.

Basford (1986) compared the effectiveness of He-Ne laser (632.8 nm) irradiation with ultra violet radiation (254 nm), on exposed and occluded full thickness wounds in pigs. Laser treated wounds received a dose of 54 mW daily, while UV treated wounds were given two minimal erythemal dose treatments bi-daily. Treatment was for 6 days per week until clinically healed. The laser treated wounds healed faster, 20.3 $\pm$ 0.9 days, than the exposed control wounds 21.1 $\pm$ 2 days. It should be noted that this is only a 5% improvement. The results also showed that the occluded, dressed wounds healed significantly faster than the controls.

Saperia *et al* (1986) irradiated full thickness wounds on the backs of pigs with He-Ne laser and demonstrated that the elevated type I and III pro collagen mRNA concentrations in the wound were markedly increased on days 17 and 28 when compared with controls. To determine the concentrations they measured molecular hybridisation with cDNA probes. Saperia concluded that laser stimulates wound healing by enhancing procollagen gene expression.

Tendon regeneration was studied in 2 groups of animals, rats (Kokino *et al*, 1985) and rabbits (Enwemeka *et al*, 1990). Achilles tendons in rats were incised and irradiated daily with He-Ne laser and an increased rate of healing resulted.. The dosage

information is not available. Enwemeka (1990) also used He-Ne laser to compare size, tensile strength and strain after irradiation of 1, 2, 3, 4, 5 mJ/cm^2 daily to the wounded tendon but found no difference between lasered tendon and controls except that fibroblasts and collagen were well aligned following laser treatment. The laser treated tendons were also smaller and the healing rate was increased. Based on other reports, the doses used were rather low to produce a beneficial result.

Enwemeka (1992) further studied tenotomized and surgically repaired rabbit calcaneal tendon. He-Ne laser irradiation daily of an unspecified dose, for 21 days, accelerated collagen synthesis. Vacuolar fibrils were induced only in fibroblasts of tenotomized, laser irradiated tendons and not in non-tenotomized, laser-irradiated tendon or non-irradiated tendons whether tenotomized or not. These results suggest that He-Ne laser can induce vascuolar fibrils, an indication of accelerated collagen turnover.

Dyson and Young (1985, 1986) lasered full thickness skin wounds in mice using combined pulsed infrared and continuous He-Ne laser to study the effect on wound contraction and wound bed cellularity of two different frequencies. Treatment time was 15 minutes, and 9 treatments at 120 mm from the wound were carried out. Treatment at 700 Hz frequency increased wound contraction and fibroblast and myofibroblast proliferation. However IR pulse frequency 1 200 Hz tended to inhibit wound contraction, although fibroblast numbers did not fall significantly. This suggests reduced contraction maybe due to some effect on the cell's contractile mechanism. The control group exhibited an irregular arrangement of fibroblasts, as did the 1 200

Hz laser-group's. The 700 Hz laser group's fibroblasts were aligned virtually parallel to each other.

Trelles *et al* (1986) irradiated the tongues of mice with a He-Ne laser (632nm), energy density 2.4J/cm^2 using 2 output powers, 4mW and 15mW with time adjusted to maintain a constant energy density. The irradiated area was 1cm^2. A single dose of 2.4 J/cm^2 produced vasodilatation and active mast cell degranulation. The higher power density, delivered faster was more efficient than the slower delivery of L.I.L.T., with degranulation occurring faster in the group irradiated with 15mW than in the 4mW group. This may have a beneficial effect on the rate of tissue repair and wound-healing.

Rochkind *et al* (1989) studied the effects of He-Ne laser irradiation on bilateral wounds and burns in rats and on the peripheral and central nervous system over a 21 day period. Wound area, action potentials and neurone degeneration were compared. Laser parameters were 7.6 J/cm^2 for the wound treatment, at 16mW for 7 min daily up to 21 days. Wound recovery was found to be enhanced on both the treated side and on the contralateral non-lasered side, which served as the control wound. The mechanism of this systemic effect of the laser is uncertain. Assessment was made by clinical observation and photography.

In the study on the effect of laser on crushed sciatic nerves, the amplitude of action potentials were increased in the crushed nerves which were irradiated, as well as in the opposite non-irradiated controls legs. Laser treatment greatly reduced motor neurone degeneration compared to the control group.

Takeda, (1988) investigated the irradiation effects of Gallium Arsenide semi-conductor laser treatment on alveolar bone after tooth extraction in rats. The results suggested an enhanced bone wound healing with increased fibroblast numbers and formation of trabecular, osteoid tissue in the lasered group.

Asencio-Arana (1988) used He-Ne laser to stimulate healing surgical colorectal anastomoses in the rat. Results showed a significant increase in strength at the anastomosis site of irradiated animals compared to non-irradiated specimens. The laser did not cause luminal narrowing in the experimental group.

Braverman *et al* (1989) and Hall (1994) reported no increases in wound healing rates in rats and rabbits. Few dosimetry details were available for Hall's study on rat wounds. Braverman studied 72 wounds in rabbits which were irradiated with either He-Ne (632.8 nm), pulsed IR (904nm) laser or combined laser treatments. Dorsal skin wounds were treated over 21 days. When compared with control untreated animals, all laser treated groups showed increased tensile strength in both the treated and untreated wounds. The wound healing rate was however unchanged. The findings on the contralateral untreated side were explained by the possible release of tissue factors such as prostaglandins and growth factors into the systemic circulation.

3.4 HUMAN STUDIES

Although several studies have been done and most claim enhanced wound healing, optimal dosimetry has not been substantiated on human subjects.

Baxter *et al* (1991) sent out a questionnaire to establish the most favoured treatment modality in physiotherapy departments in Northern Ireland. Sixty-two percent of physiotherapists used laser as their treatment of choice for chronic, trophic, diabetic and decubitus ulcers, burns, post-operative wounds and necrotic tissue. Their reason for the choice of laser was that positive results were obtained with laser. Other uses are for traumatic injuries, dental and dermatological lesions, infective sinusitis, pain relief, neuralgia's and trigger point therapy. This review will be confined to wound healing.

Mester (1963) pioneered many studies over a 25 year period. Mester *et al* (1985) stated that low energy density radiation had a stimulatory effect on cells and stimulated non healing ulcers. High energy density radiation however had an inhibitory effect. Investigating 1 000 patients with chronic, non-healing leg ulcers, he found that L.I.L.T with an energy density of $4J/cm^2$ achieved healing rates of 50 - 100% (Mester and Mester, 1989).

Several other authors report improved wound healing in ulcers (Waidelich, 1983; Mussigang, 1983; and Sugrue *et al*, 1990). Unfortunately most reports fail to provide full details of treatment times and the duration of treatment course.

Sugrue *et al* (1990) did a pilot study on 12 patients with intractable venous ulceration. The treatment duration period was 3 times a week for 12 weeks. The patients had a maximum treatment of 20 minutes duration. Two laser systems, a continuous wave Endolaser Ga AlAs, 780 nm, and a pulsed wave Space midlaser IR GaAs, 904 nm, were used for treatment. Continuous wave treatment was at 0-2 mW and pulsed treatment at 4 000 Hz, and 3mW. The results were that 2 ulcers healed completely, and the balance demonstrated a 27% reduction on ulcer size, with a 44% increase in granulation tissue and epidermal growth. In one patient, increased capillary density was noted. A significant reduction in pain from 7.5 to 3.5 on a linear analogue scale was reported. Laser irradiation had no effect on $TcPO_2$ measurement, the number of skin capillaries, or peri-capillary fibrin deposition in the lipodermatosclerotic area around the ulcer.

In a study of 6 patients with chronic leg ulcers, Crouse and Malherbe (1988), compared laser to ultraviolet light. Laser reduced wound area by 50% while ultraviolet light resulted in a 33% reduction. The results of this study were not statistically significant as the sample size of 6 patients was probably insufficient.

De Cuyper (1985) had favourable results when treating sprained ankles with L.I.L.T. De Bie and Steenbruggen (1989) also treated acute sprained ankles with an Endolaser

465 infra-red, 780 nm probe, from 48 hours post injury. The injuries were Grade 1 or 2 injuries to the anterior talo-fibular or lateral calcaneo-fibular ligaments. Treatment parameters were standardised to 180sec treatment time, a pulse energy of 5mW, a pulse frequency of 80Hz, a pulse time 0,3m sec, beam diameter of 1 mm and a skin-probe distance of 1cm. Twelve patients received laser therapy, 12 patients served as a placebo group and an untreated group of 15 patients served as a control. Ten treatments were given and pain scores were reviewed after 5 days. The laser treated group showed a significant reduction in pain. They also returned to daily activities and sport a little earlier than the other 2 groups. Couman's taping was additionally applied to all 3 groups.

Khurshudian (1989), treated 174 patients with one dose of 4.5 J/cm^2 Helium cadmium (He-Ca) laser (441.6nm) with "suppurative disease" in patients with Diabetes Mellitus. They found rapid abatement of the inflammatory response, cleansing and accelerated regeneration in the purulent wounds and found that wound healing was highly efficient in the irradiated groups when compared to a control group. They report the healing time to be "reduced by 1.5 times" in the irradiated patients.

Gostischen *et al* (1987) used the He-Ca laser in the treatment of 372 patients with purulent wounds, and found that laser stimulated wound healing. 66.8% of patients were discharged with completely healed wounds after 13.1 days.

Duodenko *et al* (1989) examined the treatment of acute lower limb thrombophlebitis with He-Ne laser irradiation. They found that the laser produced a marked analgesic,

desensitising, anticoagulative and immuno-stimulatory effect, with increased micro-circulation and oxygenation of the involved tissue. Shortened wound healing time resulted in their conviction that this should be the treatment of choice for trophic leg ulcers.

Ternovoi *et al* (1989) treated pyonecrotic, poorly granulating arm and leg wounds of 158 patients with L.I.L.T. He found that 75% healed and that wound healing time was reduced 1.5 - 2 times when compared with other traditional treatment methods.

Nussbaum *et al* (1994) compared the wound healing effect of laser treatments on pressure ulcers in 22 spinal cord injured patients. One group received routine nursing care of bi-daily moist wound dressings and continuous pressure care, the second received laser treatment and nursing care, while the third group received ultraviolet light and ultrasound (UV/US) and nursing care. The laser protocol consisted of 3 treatments weekly using a cluster probe with a 820 nm laser diode and 30 S.L.D's (10 each at 660, 880 and 950 nm) at an energy density of 4 J/cm^2, and a pulse repetition rate of 5 000 pulses/second.

The UV/US regimen consisted of 5 treatments weekly alternating the treatment modality daily. The pulsed US was applied at a frequency of 3 MHz, at 0.2 W/cm^2 (1:4 pulse ratio) for 5 min/$5cm^2$ of wound area. The UV dosage level was E1 for clean/granulating areas, E3 for purulent/slow granulating areas, E4 for heavily infected areas and 2E4 for wound debridement. Weekly percentage changes in wound area were compared. Results showed U/S / UV treatment to be the most effective.

Karu (1985) found that phototherapy was 85% successful in healing gastric and duodenal ulcers. However there was no difference in results obtained with coherent or incoherent, monochromatic light. In addition she reported that higher doses are necessary *in vivo* to produce the same effects obtained *in vitro*.

Kovacs (1981) investigated the use of He-Ne laser in cervical erosions and ectopium. Dosimetry was 5 mW, 632.8nm, at 124 cm distance from treatment site, and a $1cm^2$ irradiation area. He reported a 90 % rapid increase in epidermal growth and connective tissue metabolism. Kovacs saw this method as useful in outpatient treatment of ectopium.

Other wounds treated with laser include burns, amputation injuries, skin grafts, infected wounds and trapping injuries (Cabrero *et al* 1985). This author found that laser therapy increased both the rate and quality of healing in all cases, and especially more so in young versus old patients. The same group showed that as the healing rate increased, bacterial culture decreased, suggesting a selective bio-inhibitory effect upon wound infection. In open wounds lacking epidermis and dermal layers, treatment could theoretically be at lower energy densities. However, to avoid infection and patient discomfort, a non contact method is usually used. This decreases the energy density on the target tissue (Baxter, 1994).

Oshiro and Calderhead, (1990) highlight some additional factors which require consideration when planning laser treatment. One of these is the state of the circulation which needs careful consideration as blood proteins absorb light within the therapeutic

wavelengths. The state of the skin is also important as shiny skin will reflect more light thus increasing the energy density required to obtain optimal treatment.

CHAPTER 4.

MATERIALS AND METHODS:

All cell culture consumables were supplied by Polychem S.A (Durban) unless otherwise stated.

4.1 CELL CULTURE STUDY

4.1.1 MATERIALS:

Commercially available Eagle's Minimum Essential Medium (EMEM) supplemented with Earle's Salts, Hepes, non-essential amino acids and L-glutamine was routinely used. In addition heat inactivated foetal calf serum (FCS), antibiotics (penicillin, streptomycin and fungizone), Hank's balanced salt solution (HBSS) and trypsin were used.

4.1.2 METHODS:

Human fibroblasts obtained from the Department of Virology, University of Natal Medical School, were cultured in 25ml flasks. Flasks that contained cells with a 20-30% confluency required a routine medium change supplemented with 5 % FCS. The cells were routinely passaged as follows:

Cultured flasks containing medium with depleted nutrients were aspirated together with loosely attached cells. These flasks were then rinsed with HBSS (10ml) at 37°C

to remove cellular debris and further washed twice in HBSS. All cell culture procedures were carried out under strict aseptic conditions using a laminar flow.

Cell detachment was detected microscopically or viewed with the naked eye (detached cells gave the medium a cloudy appearance). 5ml Culture medium containing 5% antibiotics was then added and the flasks were returned to the incubator (37°C).

The fibroblasts were confluent after 3 days of subculture. The medium from culture flasks containing confluent fibroblasts was discarded and the cells were washed twice with HBSS.

A 1ml trypsin solution (0.25 %) was added to the confluent flasks and trypsin digestion was allowed to proceed for approximately 1 to 5 minutes until microscopic observation showed the cells to have rounded off slightly but not to have become dislodged from the surface. The trypsin was then removed with a sterile Pasteur pipette. Culture medium (1.5ml) containing 10 % FCS was added to the flask and the cells were dispersed in the solution by tapping the flask firmly against the hand. The resultant suspension was repeatedly pipetted with a sterile pipette to separate clumps of cells. To the 0,5ml cell suspension was added 4 ml of medium for a 1:3 split.

Culture flasks (50ml) were then stoppered and incubated at 37°C without further disturbance until the cells attached to the substrate. At this stage some fibroblasts were stored by freezing. These cells were suspended in freezing medium (culture medium

and 10 % FCS and 10 % Dimethylsulfoxide). The cell suspension (1ml) was dispensed in 2 ml vials and stored at -70°C.

Confluent fibroblast cells were trypsinized and resuspended in culture medium to give a cell count of 11 x10^4 cells/ml. A haemocytometer was used for the cell count.

This cell suspension was used to fill each of the wells of a 96 well microtiter plate with 100 µl of cell suspension per well. 200 µl Culture medium was added to each well. Control wells also contained 100 µl of cell suspension and 200 µl of culture medium. The microtiter plate was incubated for 24 hours at 37 °C.

4.1.3 CELL IRRADIATION METHODOLOGY

Each well was then irradiated three times on consecutive days ie, at 24, 48 and 72 hours post plating of the fibroblasts. A GaAlAs Endolaser 476, infrared (Class3B), continuous, low intensity laser was used. The wavelength was 830 nm and the average power output of the laser was 30 mW.

The laser probe was swabbed with absolute alcohol and aseptic conditions were strictly adhered to when treating the cell culture.

The laser probe was held perpendicular to, and steady at, the "mouth" of each well at a constant distance of 1 cm from the fibroblast layer on the base of each well. Each column (1-12) was irradiated with a pre-set dose measured in J/cm^2. Columns 11 and

12 served as controls and were not irradiated. The Endolaser probe is fitted with a target light to clearly indicate the treatment area and protective eye goggles were worn. Each dose was preset on the Endolaser, with the laser unit automatically setting the correct time according to the selected output. A continuous, 100 % output was selected.

Only 1 well was exposed at a time, the other wells were screened with tinfoil to prevent accidental irradiation. Culture medium was renewed daily from each well, and the plate was incubated at 37°C. Each of the 3 irradiations was performed at the same time daily. Each column of 8 wells was irradiated with a specific dose already stated.

4.1.3.1 STUDY DOSES

Dosages selected were 0.05, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 3, 4, and 5 J/cm^2. After completion of this experiment it was found that an error had been made in calculating the dosages delivered, as the area of each well was calculated to be 0.3318 cm^2 and not 1cm^2. The delivered dosages were thus 0.15, 0.6, 1.2, 1.8, 2.4, 3, 4.5, 9, 12, and 15 J/cm^2.

This experiment was repeated using dosages 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2.3, 3, 4, and 5 J/cm^2 for each set of wells. The dosages were calculated on a well area of 0.3318 cm^2.

4.1.4 FIBROBLAST VIABILITY ASSAY

Cell Viability was measured using the MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] reduction assay (Hanelt *et al*, 1994). The principle of this assay is that the tetrazolium salt (yellow) is reduced to a formazan (blue) dye by the succinate dehydrogenase enzymes of the mitochondria of viable cells.

The wells were decanted and 100 µl of fresh culture medium was added together with 10 µl of MTT (10µl/100µl) and the plates were further incubated for 4 hours at 37°C. Supernatants were then aspirated and 100 µl of DMSO (dimethylsulfoxide) was added to each well to solubilise the formazan crystals and incubated for a further hour at 37°C. The optical density of each well was measured spectrophotometrically using a Bio-rad spectroreader at a wavelength of 595 nm, with a reference wavelength of 630 nm.

In the first study, several readings were invalidated because the absorbance levels were beyond the range of the instrument. This was overcome in the second study by diluting each well by a factor of 10 before taking the final absorbance readings.

4.2 WOUND HEALING MODEL METHODOLOGY

Phoenix membrane discs were purchased from Bibby Sterilin Ltd, Stone, Staffordshire, U.K. The Phoenix disc consists of a silicone rubber $5x10^{-3}$ inch thick, with an outer ring which allows 0.5 ml of media to be held within the membrane

surface. The ring stabilises the membrane surface tension, and the silicone rubber is compatible with cell growth and allows visualisation of cell growth.

Human fibroblasts were cultured as described previously. A homogenous cell suspension (1.5 ml, 11 x 10^4 cells/ml) was prepared for culturing on the Phoenix membrane discs. Six Phoenix membrane discs were placed in a six well plate. An aliqout (150µl) of the fibroblast cell suspension was pipetted onto each membrane surface in the six well plate. Culture medium (350 µl) was immediately added to the cells and the six well plate was incubated at 37°C for 1 hour to allow the fibroblasts to attach to the surface of the membranes. Thereafter more culture medium (1ml) was added to each well and the plate was further incubated at 37°C to allow for a confluent layer of cells to form on the silicone membrane surface. At 72 hours post plating, the cells were confluent. A sharp, sterile cork borer of 1cm diameter was then used to punch a hole through the middle of the membrane disc and fibroblast monolayer. Three of the six membranes were "wounded". After each membrane was wounded, the cutting edge of the cork borer was sterilised in absolute alcohol. The culture medium in each of the wells was then removed and replaced with fresh medium.

4.2.1 WOUND IRRADIATION

One hour after the fibroblast monolayer on the Phoenix membrane disc was injured, the first laser irradiation was applied, using a scanning technique from edge to edge around the wound margin. All procedures were carried out under aseptic conditions.

4.2.2 IRRADIATION PARAMETERS

An Endolaser 476, 830nm, infrared laser was used. The average power output was 30mW and the active medium was GaAlAs. Protective goggles were worn. The laser was held perpendicular to the Phoenix membrane disc just above the disc in an attempt to simulate just off-contact wound laser irradiation. The laser head was swabbed with absolute alcohol prior to irradiation. The dosimetry was 1 or 4 J/ cm^2, continuous output.

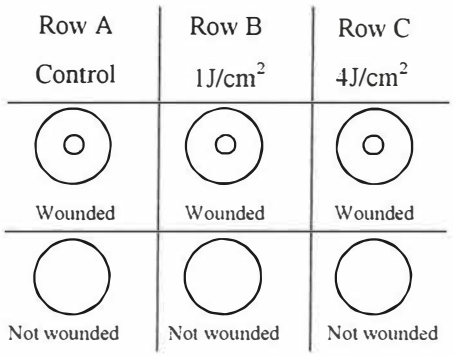

Figure 8. Six well plate with Phoenix membranes with a confluent layer of fibroblasts.

Row A served as the control and was wounded but not laser irradiated. Row B was irradiated with 1 J/cm^2 and row C was irradiated with 4 J/cm^2 (Figure 8).

The area that was lasered was scanned as described above, all the other areas being simultaneously screened with tinfoil. After irradiation, the 6 well plate was viewed microscopically and then incubated at 37°C. This procedure of irradiation was carried

out on 3 consecutive days at the same time and under aseptic conditions and then replaced in the incubator after viewing microscopically.

This experiment was carried out on two separate occasions.

4.2.3 FIBROBLAST VIABILITY ASSAY ON DISCS

Cell viability was measured using the MTT assay as described previously. A solution of MTT (30μl) was added to each well and the plate was incubated for four hours at 37°C. Once the formazan crystals were solubilised with DMSO, the coloured supernatant was aspirated and stored in a sterile 2ml vial. The absorbance of the supernatant was measured spectrophotometrically at 595nm. A solution of DMSO served as a blank.

CHAPTER 5.

RESULTS

5.1 CELL CULTURE STUDY 1.

The fibroblast cells grew rapidly and after three days in culture the cells appeared scattered on the substrate. The cells were monolayered, agranular and were tightly adherent to one another. After a week in culture, cells appeared tightly packed together (Figure 9). Many cells displayed prominent nuclei which were observed by phase contrast microscopy (x1600).

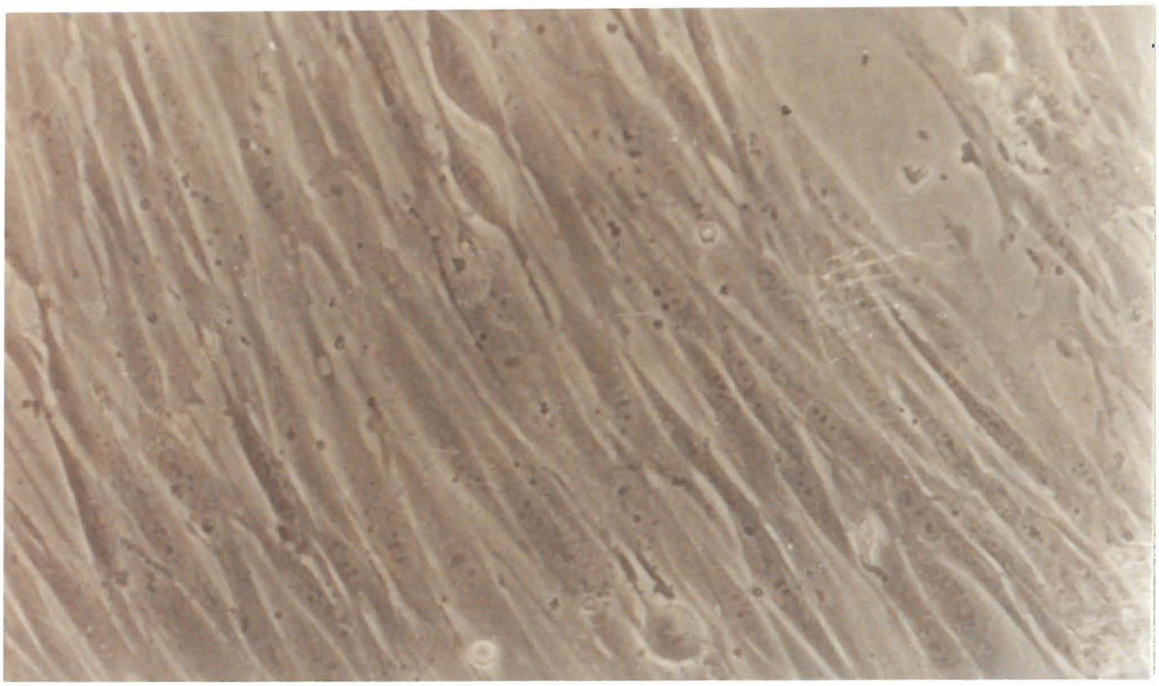

Figure 9. A phase contrast micrograph of fibroblast growth in a culture flask after 1 week in cell culture.

Unlasered fibroblasts treated with MTT appeared pink (Figure 10) which indicated viable cells. After laser treatment however, activated cells reacted with the added tetrazolium salt (MTT) to form purple crystals. DMSO solubilises the crystals and results in the production of a purple dye (formazan) (Figure 11).

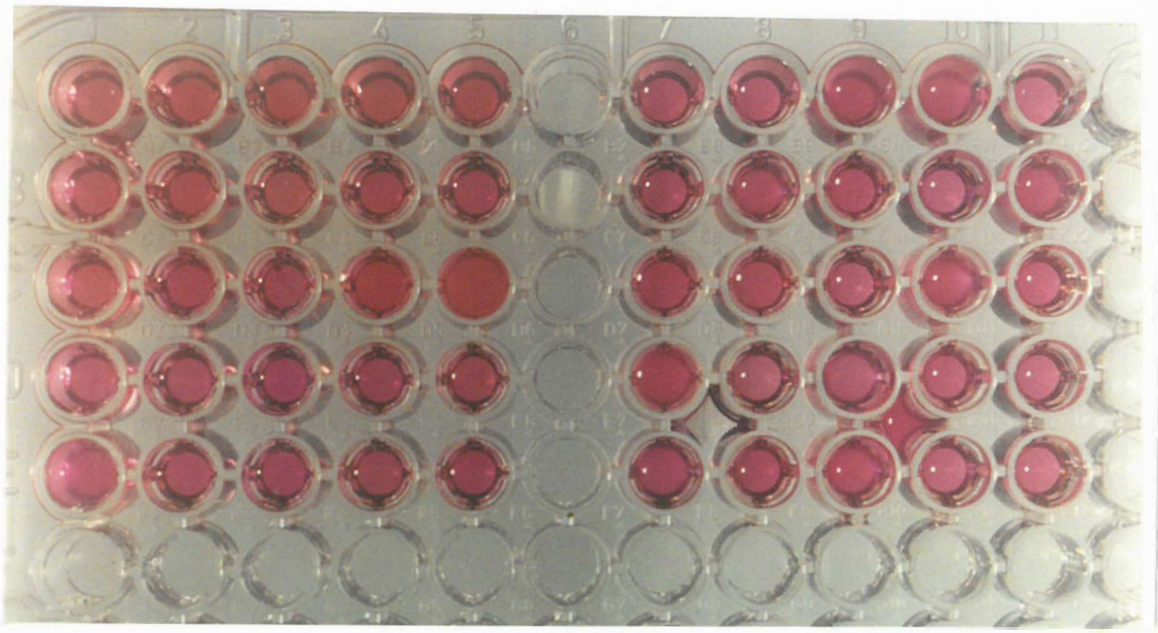

Figure 10. Unlasered fibroblasts, MTT assay.

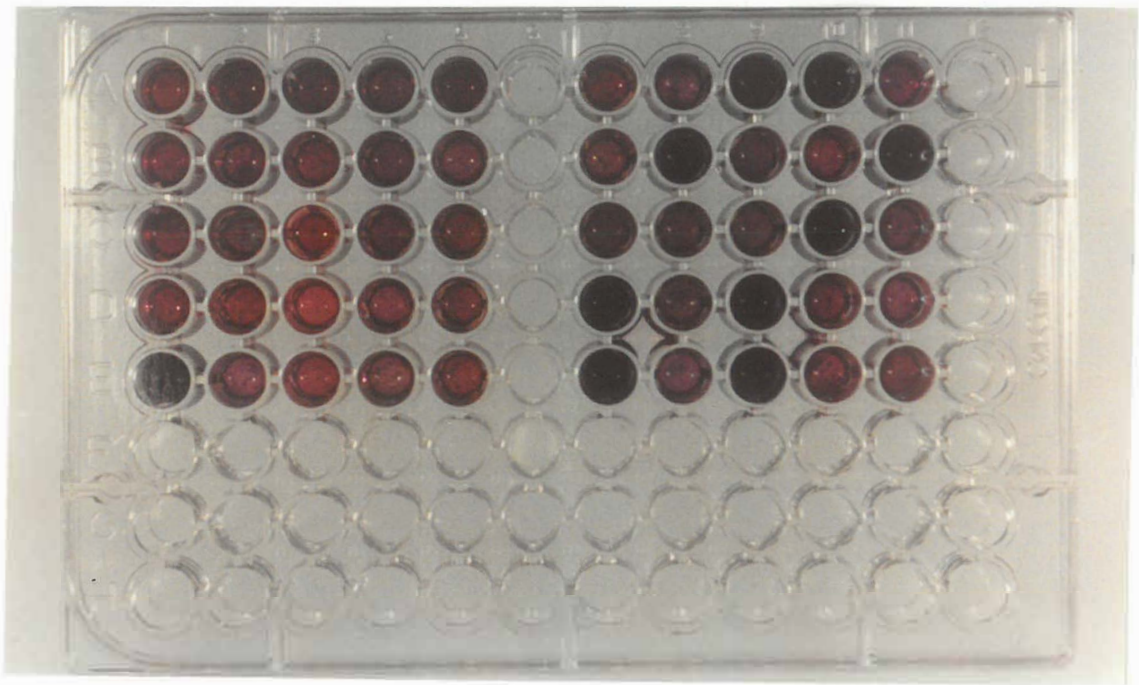

Figure 11. MTT assay results in the fomation of formazan.

The results of the MTT assays for the first study using the 96 well plates are shown in Table 1. The maximum possible absorbance reading was obtained in three wells. The true readings may in fact have been higher in these wells.

Table 1. Absorbance readings following MTT assay. Columns 11 and 12 were not lasered and served as controls.

	Dose J/cm^2											
	0.15	0.6	1.2	1.8	2.4	3.0	4.5	9.0	12.0	15.0	0	0
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.47	1.077	1.213	1.52	1.528	1.83	1.386	1.93	1.855	1.204	1.3	1.489
B	0.983	1.351	2.118	1.459	1.279	2.308	2.151	1.53	1.613	1.768	1.473	1.111
C	0.941	1.542	1.469	1.392	1.043	1.038	0.697	1.947	0.841	0.798	0.78	0.795
D	1.486	1.216	1.44	0.889	1.467	0.789	0.746	0.493	0.882	0.91	1.016	1.127
E	1.132	1.391	2.5	1.994	1.851	1.252	1.024	1.128	1.616	2.5	1.235	0.843
F	1.269	1.514	1.084	0.969	0.766	1.243	0.875	1.105	2.5	1.824	1.557	0.722
G	1.179	1.507	1.393	0.983	0.67	0.958	1.069	1.054	1.247	1.803	1.573	0.772
H	1.378	1.059	1.96	0.876	1.083	0.783	1.048	0.788	1.153	0.647	0.585	0.551
Avg	1.104	1.332	1.647	1.260	1.210	1.275	1.124	1.246	1.463	1.431	1.189	0.926
SD	0.315	0.194	0.491	0.398	0.399	0.536	0.467	0.519	0.553	0.642	0.366	0.298
COV	28.6	14.6	29.8	31.6	32.9	42	41.6	41.6	37.8	44.9	30.8	32.2

One way analysis of variance (ANOVA) shows no difference between the treatment doses ($F=1.39$ and $P=0.19$). These results were then divided into low dose, columns 1 - 5, and high dose, columns 6 - 10. ANOVA of the low dose results including the controls showed a significant difference between treatments ($F= 3.09$ and $P=0.164$). Post hoc testing using unpaired T Tests and the Bonferonni correction showed 1.2J/cm^2 to be the dose that differed significantly from the controls, ($P=0.0026$ with significance set at $P<0.01$). ANOVA of the high doses and the controls showed no differences ($F= 1.089$ and $P= 0.378$). For all ANOVA's the control results were combined.

5.2 CELL CULTURE STUDY 2.

The results of the MTT assays for the second study using the 96 well plates are shown in table 2. In this study the cells were diluted 10 times to ensure that absorbance readings were within the range of the spectrophotometer.

Table 2. Absorbance readings following MTT assay. Columns 1 and 12 were not lasered and served as controls.

	Dosage J/cm^2											
	0	0.2J	0.4J	0.6J	0.8J	1.0J	1.5J	2.3J	3J	4J	5J	0
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	0.71	1.00	0.77	1.06	1.09	0.66	0.96	0.63	1.05	1.25	1.29	0.93
B	1.12	1.46	0.92	1.11	1.11	0.87	1.18	0.44	0.52	0.95	1.84	0.97
C	0.69	0.77	1.04	1.22	0.25	0.85	0.83	1.27	0.57	0.55	1.35	0.99
D	0.61	1.44	1.71	0.96	1.16	0.44	1.27	1.04	0.42	0.95	1.66	0.9
E	0.71	1.07	1.71	0.81	0.81	0.5	0.62	0.77	0.48	0.88	0.86	1.24
F	0.62	0.87	1.04	0.94	1.06	1.11	0.31	0.65	0.58	0.72	0.53	1.03
G	0.38	0.98	1.06	0.65	1.06	1.08	0.64	0.91	0.66	1.22	1.16	0.47
H	0.73	0.72	1.05	0.68	1.05	0.56	0.63	1.11	0.97	0.77	0.92	0.82
Avg.	0.70	1.04	1.16	0.93	0.95	0.76	0.81	0.85	0.66	0.91	1.20	0.92
std	0.21	0.28	0.35	0.20	0.30	0.26	0.32	0.28	0.23	0.24	0.43	0.22
COV	29.5	26.9	30.2	21.9	31.7	33.9	39.8	32.8	35.1	26.3	35.8	23.8

ANOVA shows a significant difference between the treatment doses (F= 2.827 and P=0.004). Post hoc testing shows the doses of 0.4J/cm^2 and 5J/cm^2 to differ significantly from the controls. The co-efficient of variance ranged from 21.9% to 39.8%.

5.3 WOUND HEALING MODEL

The absorbance readings for the two studies are shown in tables 3 and 4.

Table 3. Absorbance readings for experiment 1.

	Control	1J/cm^2	4J/cm^2
Wounded	0.191	0.416	0.47
Not Wounded	1.269	1.473	1.582

Table 4. Absorbance readings for experiment 2.

	Control	1J/cm^2	4J/cm^2
Wounded	0.107	0.329	0.398
Not Wounded	1.004	1.211	1.366

The relationship of dose to change in absorbance in the wounded and unwounded discs is shown in figure 11, where the absorbance after treatment at 1J/cm^2 and 4J/cm^2 is shown as a ratio of the absorbance of the control disc.

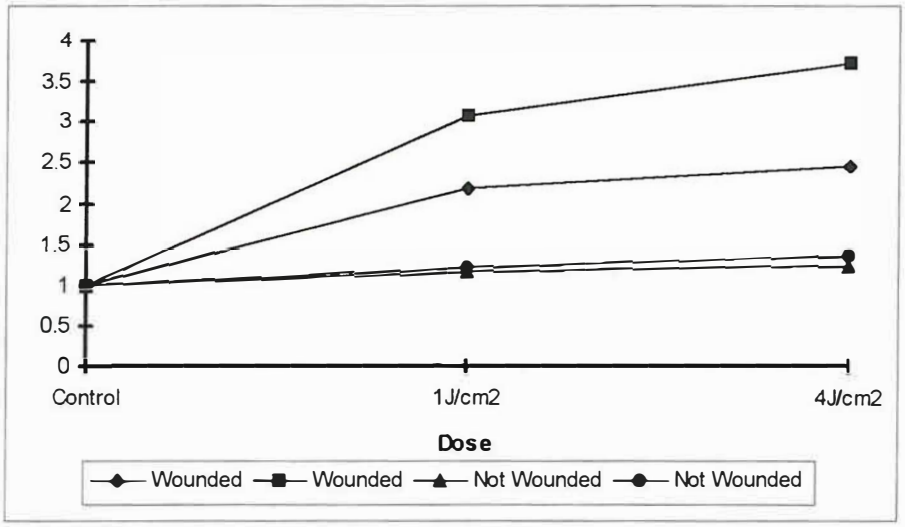

Figure 11. Effect of laser treatment on fibroblast proliferation expressed as the ratio of the absorbance of the MTT assays of treated fibroblasts to the untreated controls.

CHAPTER 6

DISCUSSION

6.1 DOSIMETRY STUDIES

The findings of the two dosimetry studies using 96 well plates show that *in vitro* irradiation of human fibroblasts using L.I.L.T. at a wavelength of 830nm, at a maximum power output of 30mW and at various energy densities can influence fibroblast proliferation. The results of the first study should be viewed with caution because of the problems encountered with the absorbance readings. In the second dosimetry study, the results suggest that at a dose of 0.4 J/cm^2 and 5 J/cm^2 maximal fibroblast proliferation was observed. Between energy densities of 0.6 J/cm^2 to 4 J/cm^2 there was no statistical evidence of an increase in cell turnover. At 1J/cm^2 and 3J/cm^2 the mean absorbance values noted was less than that of the controls.

The Arndt-Schultz Law states that theoretically there is an optimal threshold dose to achieve a biostimulatory effect in the target tissue, but that at a certain point saturation will occur and a plateau effect will occur (Baxter, 1994). Such a plateau was not seen in either of the two dosimetry studies. Bio-inhibition may also occur, and this effect may be useful for treatment of keloids and hypertrophic scars.

It could be argued that the assay used to demonstrate cell viability was inappropriate or not sensitive enough for this type of study. It was chosen because laser irradiation

causes increased ATP production in the ferric sulphide redox system in the mitochondria and in turn the increased energy production increases cell function (Karu, 1987). The MTT assay, which measures the activity of succinate dehydrogenase in the mitochondria has been used successfully in many other cell culture studies and should be sufficiently sensitive to identify changes in cell proliferation. Another possible explanation of the findings may be that the MTT assay should have been delayed until the latent biostimulatory response noted by Rajarathan *et al* (1994) occurred. Rajarathan noted no differences in proliferation at 72 hours post treatment, but significant changes at 120 hours.

Most previous studies on fibroblast proliferation have used the He-Ne laser (632.8nm) (Mester *et al*, 1978; Abergel *et al*, 1984; Tocco *et al*, 1985; Boulton and Marshall, 1986; Hallman *et al*, 1988; Pourreau-Schneider *et al*, 1990; Zugaro *et al*, 1992; Lubart *et al*, 1992; van Breugel, 1992). The He-Ne laser usually emits a non pulsed continuous waveform. Boulton and Marshall (1986) for example used a 50% pulsed He-Ne output. The semiconductor GaAlAs laser (830nm) used in this study is currently the most popular in the clinical situation (Baxter, 1994) and may be used in continuous or pulsed mode. The two studies reporting the use of the GaAlAs laser investigated its effect on macrophage stimulation of fibroblasts (Young *et al*, 1989) and on gene expression of collagen formation (Abergel *et al*, 1987). There have been no previously reported studies in the English literature on the use of continuos mode GaAlAs laser on fibroblasts.

Energy density is an unresolved issue, as most authors fail to report the exact parameters used. Knowledge of the power output of the equipment, the treatment duration, the irradiation dose and the area of irradiation are vital for comparison of experimental work. Van Breugel (1992) felt that power density as well as irradiation time was important. He found that irradiation times of 0.5 - 2 minutes to be most stimulatory, and that power outputs of 0.55 - 5.98 mW enhanced cell proliferation. Unfortunately the surface area irradiated was not stated, so the energy density is not known.

Bolton *et al* (1990, 1991) found maximal fibroblast proliferation at energy densities of 2.4 - 7.2 J/cm^2. Based on He-Ne laser wound healing studies, Mester and Jaszsagi-Nagy (1973) stated that the therapeutic treatment window was between energy densities of 1 - 4J/cm^2 and that above 4J/cm^2 a saturation effect occurred. In the present study, significant differences in fibroblast proliferation were seen at 5J/cm^2.

While the literature constantly recommends low dose laser therapy there is little evidence to support this. An unusual finding in the present study was the significant increase in proliferation of fibroblast activity at a dose as low as 0.4J/cm^2. There are very few studies to have used a dose as low as this and none on fibroblasts using a continuous waveform.

Possible limitations to this study and other *in vitro* cell culture models are the distance between the probe and the base of the cell culture wells, which in this study was 1 cm. What is not known is the absorbance capacity of the cell culture medium. Dose in cell

culture may well then be also medium dependent. Another unanswered variable is the amount of reflection and refraction of the laser light by the medium. This would obviously affect the energy dose reaching the cells. Although every attempt was made to isolate each well during treatment by screening the other wells with tin foil, some energy may have been transmitted through the plastic of the 96 well plate.

6.2 PHOENIX WOUND MODEL

The Phoenix wound model is a new attempt at replicating the process of wound healing *in vitro*. There are obvious limitations in that the inflammatory response with all the resultant humoral and cellular responses derived from other cells involved in the process are missing. It does however allow for examination of the response of one cell line to wounding, and allows a controlled environment in which to study the effect of a treatment intervention. The results of this study confirm the findings of other researchers in routine cell culture, that laser irradiation stimulates fibroblast proliferation *in vitro*.

The response of the fibroblasts to doses of 1 and 4J/cm^2 after wounding is of interest. While in the unwounded controls a 16 to 36% increase in cell proliferation was recorded after treatment, it is in the wounded discs that a more noticeable response occurs, with increases in cell proliferation ranging from 218% to 372%. The sample size is unfortunately too small to allow for statistical comparison. It is proposed that this aspect of the study be continued to increase the sample size to allow for statistical

evaluation. The Phoenix wound model may well prove to be a useful additional tool for the investigation of individual factors in wound healing.

6.3 SUMMARY

Research into the use of laser in wound healing is beset with many problems. The issues of dose (continuous or pulsed), treatment time, power output, and treatment area have been addressed. *In vitro* there are further problems such as the of the lack of the skin layer which will influence delivered dose, no information on the absorbance, refraction, reflection and scattering capacity of culture media, and the attempt to infer from the activity of one cell line in isolation its activity in a multifaceted repair process. From review of the literature there appears to sufficient evidence supporting the benefits of laser. The exact nature of its beneficial action and the optimal dosages still await elucidation. The use of the wound healing model may assist in investigating specific responses of individual cell lines to the wounding.

6.4 RECOMMENDATIONS FOR FURTHER STUDIES

A further study should be undertaken comparing the response of laser treated fibroblasts in culture to the response of fibroblasts activated by laser treated macrophages. In addition to MTT assay of cell viability, measurement of the response of cytokines, such as fibroblast growth factor, should be made.

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