Laser irradiation: a complementary treatment for wounds

N N Houreld[1] **and H Abrahamse**

Laser Research Centre, Faculty of Health Sciences, University of Johannesburg, P.O. Box 17011, Doornfontein, South Africa, 2028. Tel: +27 (0)11 559-6406

E-mail: nhoureld@uj.ac.za / habrahamse@uj.ac.za

Abstract. *Aim:* Since the invention of the laser, its application in the health sector has been studied and in an attempt to discover effective alternative treatments, Low Level Laser Therapy (LLLT), commonly known as biostimulation or photo-biostimulation, has emerged. This therapy has been successfully used both in *in vitro* and *in vivo* studies in wound healing. Although this therapy is in use worldwide, the mechanism of action is not fully understood. *Methods:* Various cell culture models, such as wounded, diabetic wounded, ischemic and hypoxic have been exposed to visible and infra-red laser light and the effect on cell migration, cell survival, proliferation, cytotoxicity, mitochondrial responses, nitric oxide (NO) release, secondary messenger activation, DNA damage and pro-inflammatory cytokine expression have been studied. *Results:* Laser irradiation at the correct wavelength and fluence has shown to have a positive effect on stressed cells *in vitro*. There is an increase in migration, survival and proliferation, mitochondrial activity, NO release and secondary messenger activation. A decrease in cytotoxicity, DNA damage and pro-inflammatory cytokines is also seen. *Conclusion:* LLLT offers an alternative wound healing therapy. At a biochemical level there is a positive effect on cells, with stressed cells being pushed into cell survival pathways.

1. Introduction

Wound healing involves a series of overlapping and intertwining events all aimed at reversing the loss of structural integrity and is controlled by a wide variety of cells, growth factors, cytokines and enzymes, all of which are released at the wound site [1]. Wound healing is divided into four main events, namely haemostasis, inflammation, proliferation and remodelling. When this sequence of events is disrupted, delayed wound healing ensues. Chronic, slow-to-heal or non-healing wounds are a common complication of diabetes mellitus (DM), particularly on the feet and lower limbs. These diabetic foot ulcers are susceptible to infection and often necessitate lower-limb amputation, which impacts heavily on patients, their families, health care departments and government. Around 20% of all patients with DM who develop foot ulcers require amputation [2]. Amputation occurs 30 times more frequently in diabetic patients than in the general population [3]. The underlying causes of these foot ulcers are thought to be due to micro- and macrovascular disease and advanced glycation end products [4] and are associated with sensory loss. The current treatment protocol is both systemic, treatment of the metabolic condition (diabetes), and local treatment of the diabetic ulcer. Common treatment of the diabetic foot involves debridement, wound care and dressing, antibiotic therapy,

[1] To whom any correspondence should be addressed.

offloading, accommodative orthodontics and wearing the correct footwear. Patients are encouraged to examine their feet daily and should visit their healthcare providers annually for check-ups.

Low-Intensity Laser Irradiation (LILI), also known as phototherapy or photobiomodulation, is the use of low-powered lasers to stimulate cellular metabolism and biochemical processes. LLLT is widely used in dermatology, somatology, dentistry, physiotherapy and veterinary science, and applications include wound healing, pain attenuation, modulation of the immune system and bone and nerve repair. Wavelengths of between 500 – 1,100 nm are commonly used with fluencies around 1-4 J/cm^2 [5]. The choice of wavelength is dependent on the depth of penetration required, and wavelengths of 600 – 700 nm are commonly used to treat superficial wounds (shorter penetration depth), while 780 – 950 nm is used for treatment of deeper tissues [6].

LILI has been show to stimulate and improve wound healing both *in vitro* and *in vivo*. Caetano and colleagues [7] showed that phototherapy (combined 660 and 890 nm, 3 J/cm^2) not only promoted the healing of large ulcers which did not respond to conventional treatments, but healing was also significantly faster in irradiated groups. Minatel and colleagues [8] also showed that combined 660 and 890 nm promoted healing in diabetic leg ulcers. Granulation and healing rates were higher in irradiated groups. Moore and co-workers [9] showed that LILI in the visible red range (665 and 675 nm; 10 J/cm^2) stimulated fibroblast and endothelial cell proliferation. Lim and co-workers [10] showed that LILI with 635 nm reduced intracellular reactive oxygen species (ROS), an important aspect in angiogenesis, and an increase in viability in hypoxic/ischemic endothelial cells. LILI has also been shown to be effective in the treatment of infected wounds [11,12].

2. Methods

Fibroblasts (WS1, ATCC, CRL-1502) have been used in wound healing studies in a variety of cell models. These include normal, wounded (scratch model), diabetic (continuous growth in additional glucose), diabetic wounded, acidic (pH 6.70), hypoxic (oxygen deprivation) and ischemic (oxygen and foetal bovine serum, FBS, deprivation) and have been previously described [13-16]. Irradiation parameters are shown in table 1, and cellular assays to monitor changes in irradiated cells are shown in table 2. Cells were irradiated in the dark from the top without culture lids (figure 1). A non-irradiated control (0 J/cm^2) was included for each model in each experiment and treated in the same manner, barring irradiation; instead they were placed in a dark box on the bench. Cells were irradiated with 0, 0.5, 2.5, 5, 10 or 16 J/cm^2. Post-irradiation cells were incubated for a variable amount of time and cells detached by trypsinization and re-suspended to a concentration of 1 x 10^5 / 100 μl.

Table 1. Laser parameters.

	Laser			
Parameter	He-Ne	Diode		Nd:YAG
Wavelength (nm)	632.8	830	636	1064
Wave emission	Continuous	Continuous	Continuous	Continuous
Power output (mW)	23	55	95	1,000
Power density (mW/cm^2)	2 - 3	6	11	12.7
Spot size (cm^2)	9.1	9.1	9.1	78.5

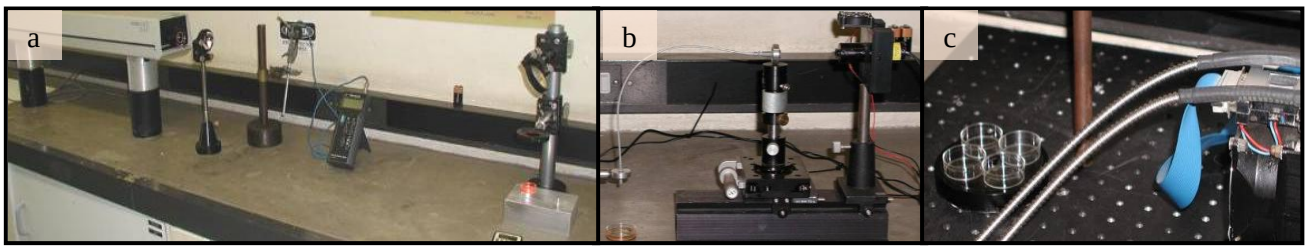

Figure 1. Laser irradiation of cells using a He-Ne laser (a), 830 nm diode (b) and Nd:YAG laser (c).

Table 2. Assays used to measure cellular responses to LILI.

Parameter	Assay	Data Collection
Morphology	Migration, haptotaxis	Light microscopy
Viability	Trypan blue staining	Light microscopy
	Adenosine Triphosphate (ATP)	Luminescence
Proliferation	Optical density (OD)	Spectroscopy (A_{540} nm)
	Basic Fibroblast Growth Factor (bFGF)	ELISA (A_{450} nm)
	Alkaline Phosphatase (ALP)	Colorimetric (A_{405} nm)
	XTT (tetrazolium salt)	Colorimetric (A_{450} nm)
Cytotoxicity	Lactate Dehydrogenase (LDH)	Colorimetric (A_{490} nm)
Apoptosis	Caspase 3/7	Luminescence
DNA Damage	Comet assay	Fluorescent microscopy
	Real Time RT-PCR	Fluorescence
NO	Griess assay	Colorimetric (A_{540} nm)
Cytokines	Interleukin (IL)-6	ELISA ($A_{450/\ 570}$ nm)
	IL-1β	ELISA (A_{450} nm)
	Tumour Necrosis Factor alpha (TNF-α)	ELISA (A_{450} nm)
Secondary messengers	Cyclic Adenosine Monophosphate (cAMP)	Colorimetric (A_{405} nm)
	Intracellular calcium (Ca^{2+})	Colorimetric (A_{612} nm)
Mitochondrial responses	Mitochondrial Membrane Potential (MMP)	Flow Cytometry
	Enzyme kinetics	Spectroscopy

3. Results and Discussion

All results are summarised in table 3.

3.1. He-Ne laser (632.8 nm)

Hawkins and Abrahamse [13,17] showed that when normal and wounded fibroblast cells were irradiated at 632.8 nm, 2.5 J/cm^2 increased cellular proliferation and had no adverse effect on cellular viability, DNA damage or cytotoxicity. When the same cells were irradiated with 5 J/cm^2, cells migrated towards the central scratch and there was an increase in viability and proliferation. On the other hand, 10 and 16 J/cm^2 produced a significant amount of cellular and molecular damage to cells. Morphologically, there was evidence of cellular lysis with no migration and cells detaching from the culture dish. There was a decrease in viability and proliferation and an increase in cytotoxicity and genetic damage. Wounded cells responded better when irradiated once a day on two consecutive days (24 h between irradiations). The effect on proliferation was further validated by incubating cells in the presence of 5 mM hydroxyurea (HU), an inhibitor of proliferation. In the presence of HU, irradiation with 5 J/cm^2 was still able to increase proliferation and migration of wounded cells [18].

Diabetic wounded cells irradiated at 5 J/cm^2 showed significant increases in migration, proliferation and IL-6 (a marker of proliferation and differentiation), while 16 J/cm^2 gave the opposite results [19]. Cells responded better when irradiated on two non-consecutive days (72 h between irradiations) [14,20,21]. There was complete closure of the central scratch when irradiated with 5 J/cm^2. In fact, there was a decrease in DNA damage and membrane damage (LDH released by damaged, compromised cells) as compared to non-irradiated cells. Cells irradiated on two non-consecutive days with 16 J/cm^2 still showed an increase in damage and incomplete wound closure.

The induction of acidosis and hypoxia significantly reduced MMP. When irradiated with 5 J/cm^2, there was an increase in Ca^{2+} which produced an increase in MMP, ATP and cAMP in wounded, acidotic and hypoxic cells, while as expected; a fluence of 16 J/cm^2 produced a decrease in MMP [22]. Thus laser irradiation was shown to restore cellular homeostasis, even in stressed cells (diabetic, hypoxic, acidotic) by increasing mitochondrial activity which in turn stimulates a variety of other cellular functions.

Table 3. Summary of results.

	Laser (nm)							
	632.8		636		830		1064	
Parameter	5 J/cm^2	16 J/cm^2	5 J/cm^2	16 J/cm^2	5 J/cm^2	16 J/cm^2	5 J/cm^2	16 J/cm^2
Morphology (migration)	↑	↓	↑	↓	↑	↓	↓	↓
Viability	↑	↓	↑	↓	↑	↓	↓	↓
Proliferation	↑	↓	↑	↓	↑	↓	↓	↓
Cytotoxicity	↓	↑	↓	↑	↓	↑	↑	↑
Apoptosis	↓	↑	↓	↑	↓	↑	↑	↑
DNA Damage	↓	↑	↓	↓	↓	↑	↑	↑
NO	n/a	n/a	n/a	n/a	↑	n/a	n/a	n/a
Inflammatory Cytokines	n/a	n/a	↓	n/a	↓	n/a	n/a	n/a
Secondary messengers	↑	↓	n/a	n/a	n/a	n/a	n/a	n/a
Mitochondrial responses	↑	↓	↑	n/a	↑	n/a	n/a	n/a

↑ - Increase ↓ - Decrease

3.2. Diode laser (830 nm)

Wounded cells irradiated with 5 J/cm^2 showed increased migration of cells across the central scratch as compared to unirradiated cells [23]. Cells also showed an increase in proliferation (IL-6).

Diabetic wounded cells irradiated at a longer wavelength in the infra-red range also responded to 830 nm when irradiated with 5 J/cm^2 on two non-consecutive days [24,25]. There was an increase in migration and hastened wound closure; however closure of the wound was incomplete as seen in the same cells irradiated at 632.8 nm, an increase in proliferation and a decrease in cellular and genetic damage was also seen. Cells irradiated with 16 J/cm^2 showed more damage compared to non-irradiated and irradiated (5 J/cm^2) cells. A fluence of 5 J/cm^2 was able to increase NO and ROS, and produce a decrease in the inflammatory cytokine TNF-α and IL-1β [1]. The increase in NO appears to be due to a direct photochemical process since the increase is seen immediately and not at 1 h post-irradiation. Thus, laser irradiation has an anti-inflammatory response on diabetic wounded cells.

3.3. Diode laser (636 nm)

As with irradiation with the He-Ne laser, irradiation at 636 nm with a diode laser produced an increase in DNA damage in wounded cells irradiated with 5 or 16 J/cm^2. However, when cells were left to incubate for 24 h post-irradiation, a significant decrease was seen compared to the same cells incubated for only 1 h [26]. Thus the initial increase in DNA damage is repairable and DNA mechanisms are activated. When real time reverse transcription (RT) polymerase chain reaction (PCR) was used to determine the expression of the DNA repair enzyme N-methylpurine DNA glycosylase (MPG; repairs modified bases including 8-oxyguanine, which is detected by the modified comet assay using enzymes), no significant difference in expression was detected. Thus DNA repair is by a mechanism other than MPG [26].

At 5 J/cm^2 irradiation increased cellular viability and proliferation and decreased apoptosis in hypoxic cells [16]. There was also a decrease in the pro-inflammatory cytokine TNF-α. A decrease in IL-1β and apoptosis was seen in diabetic wounded cells, as was an increase in viability and proliferation.

3.4. Nd:YAG (1064 nm)

With irradiation using near infrared laser light, both wounded and diabetic wounded cells irradiated at 1064 nm with 5 or 16 J/cm^2 showed a significant decrease in migration, viability and proliferation [23-25,27]. Diabetic wounded cells showed an increase in damage and apoptosis at both fluencies [24,25], while normal wounded cells did not show an increase at 5 J/cm^2 [23].

4. Conclusion

Stressed cells (wounded, diabetic, diabetic wounded, hypoxic, ischemic, acidic) showed significant differences as compared to normal cells, showing that these models are effective to use in wound healing studies, as well as LILI studies [15]. The effect of LILI, or any other treatment, may show insignificant effects on normal, non-stressed cells. Thus it is important to induce some sort of stress onto cells when conducting these kinds of experiments, as well as testing these models against normal cells.

LILI stimulates cells in a dose, wavelength and cell model dependent manner. The higher the dose and wavelength, the more damage is inflicted on cells. The more stressed cells are, the better they respond to the laser irradiation. It is thus important to choose the correct laser parameters, otherwise results may be interpreted as negative or having no effect due to the incorrect factors. Although a dose of 16 J/cm^2 may not appear to be very high, it must be remembered that cells are irradiated within a confined environment. The energy absorbed by the cells cannot be dispersed as occurs *in vitro* [14]. Cells irradiated at 1064 nm showed the most damage compared to cells irradiated at shorter wavelengths. This damage may be due to the generation of ROS above the threshold [24]. This difference may also be due to the different output powers of the different lasers, as lower irradiances have been found to be more effective [25]. Visible and near-infrared light is absorbed by different chromophores, or light absorbing components within the cell. Visible light has been found to directly stimulate the mitochondria, while near-infrared light affects membrane channels [28]. Cells irradiated at 632.8 nm and 830 nm was more effective than the same cells irradiated at 1064 nm. The time of incubation post-irradiation can also play a part in the results seen, and should be chosen with care [27,29] as an increase can initially be seen, but with time can change to a decrease.

LILI is able to stimulate cells in a positive manner and has been shown to be effective in wound healing *in vivo* [8]. The underlying effects for this healing are complex and intertwined. LILI stimulates mitochondrial activity [22], which stimulates secondary mechanisms which ultimately leads to normalisation of cellular function. When using the correct parameters, it protects cells from apoptosis and damage [25]. It has an anti-inflammatory effect [1,16] and directs cells into a cell survival pathway.

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