Inflammatory response of injured diabetic fibroblasts after low intensity laser irradiation at a wavelength of 830 nm

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Abstract. *Introduction*: Diabetes mellitus (DM) is a chronic disease characterized by impeded glucose metabolism and preceded by diabetic ulcers which are chronic due to deteriorated healing processes. Hypoxia, decreased fibroblast proliferation and impaired growth factors are amongst root factors that contribute to impaired healing. Photostimulation is a non-invasive treatment that utilizes low intensity laser irradiation (LILI) to stimulate appropriate cellular functions. *Materials and Methods*: Human skin fibroblast cells (WS1) were used in this study that consisted of four groups viz. normal, normal wounded, diabetic wounded and hypoxic, each with a non-irradiated control. Wounding was simulated by creating a central scratch using a pipette. A diabetic state was induced by growing cells in media that contained excess glucose to a final concentration of 22.56 mM, and for hypoxic insult, cells were incubated under anaerobic conditions (0% O_2 and 20% CO_2) for 4 h. Cells were then irradiated at a wavelength of 830 nm with 5 J/cm^2 and incubated for 1 or 24 h. Morphological changes were observed by light microscopy; ELISA and flow cytometry were used to determine interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α as inflammatory markers; and caspase 3/7 for apoptosis was determined by luminescence. *Results*: After a 24 h incubation period the wounded area appeared decreased and hypoxic cells had regained normal morphologic features when irradiated, TNF-α and IL-1β had decreased in irradiated samples, whereas IL-6 was increased. Caspase 3/7 had decreased in irradiated samples at both 1 and 24 h. *Conclusion*: This study demonstrated the beneficial effects of LILI since the results showed significantly reduced inflammatory responses *in vitro* and hastened wound healing particularly under diabetic and hypoxic conditions.

1. Introduction

Diabetes mellitus (DM) is a group of chronic diseases with diverse etiology which are characterized by metabolic changes and chronic hyperglycaemia as a result of insulin deficiency [1]. The incidence of diabetes has increased in the past decade due to increased obesity often due to incorrect nutrition, changes in lifestyle and durability or physical inactivity [2,3]. The consequences of insulin deficiency lead to abnormal lipid, protein and carbohydrate metabolism as well as coronary vascular diseases (CVD) which attributes to 50-80% of deaths and usually affect every organ system in the body including the skin [4].

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Diabetic patients with extensive duration of metabolic abnormalities are prone to complications such as vascular diseases, stroke and ischeamic heart conditions (macrovascular disorders) as well as nephropathy, neuropathy and retinopathy (microvascular disorders) [5,6]. Hypoxia is a decrease or deficiency in oxygen supply to the tissue. Diabetic patients tend to suffer from low vascular oxygen diffusion thereby inducing hypoxic insults [7,8].

Chronic foot ulcers or diabetic wounds, which are lesions resulting from skin breakage, are common in diabetic patients. Diabetic wounds are chronic since the healing process is impaired as determined clinically and experimentally [9]. They are also difficult to treat and usually prone to infection which may lead to gangrene due to ineffective wound closure [10]. Factors such as ischemia/hypoxia, decreased fibroblast proliferation or migration and impaired growth factors contribute to impaired wound healing [11]. Wounding initiates the expression of multi-factorial cytokines and growth factors which have ample activities that render them attractive agents for stimulating tissue repair. Cytokines and growth factors are signalling molecules expressed in response to activating stimuli such as tissue damage. Inflammatory markers such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) have been associated with diabetes [12].

Low intensity laser irradiation (LILI) or therapy has been a subject of interest in recent decades and is due to its desirable effects, particularly photo-biostimulation, when applied properly in addition to being non-invasive. It has been documented to be effective in wound closure. Exposure to light photons result in primary effects which are due to photoreception of photons with cytochromes; secondary effects which are induced by primary effects i.e. proliferation; and finally tertiary effects which are indirect because distant cells respond to changes incurred by other cells that have interacted directly with other photons [13,14]. Positive recovery of diabetic ulcers has been reported [15] in addition to reduced inflammatory markers [16]. Kawalec *et al*., [17,18] found that the healing time of diabetic wounds had reduced and both chronic (58%) and acute (100%) wounds completely healed. The aim of our study was to determine the effects of irradiation on wound closure or morphological changes and inflammatory markers *in vitro* using a fluence of 5 J/cm^2 at a wavelength of 830 nm.

2. Methods and Materials

2.1. Cell cultures

Human skin fibroblasts, WS1 (ATCC, CRL-1502) were cultured in minimum essential media (MEM; 32360026) and the media was supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin (antibiotic) and fungizone (anti-fungal), 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Cultures were maintained at 37 °C in 5% CO_2 and 85% humidity.

The study consisted of four groups viz. normal (N), normal wounded (NW), diabetic wounded (DW) and hypoxic (H) and each group had a non-irradiated (sham-irradiated) control. Diabetic insult was simulated by maintaining cells in a media containing additional glucose (17 mM) resulting in a final concentration of 22.56 mM [19,20]. Hypoxic insult was achieved by growing cells in FBS free media overnight [21-23] and incubating them in an anaerobic chamber for 4 h with anaerobic gas pack (0% O_2 and 20% CO_2 within 2 h) [24]. A wound was simulated by making a central scratch using a 1 ml pipette.

2.2. Laser irradiation

All laser irradiations were conducted in a dark room. An 830 nm diode laser was used with an output power of 45 mW. The irradiance spot size was 9.1 cm^2. Cells were irradiated for 16 min 50 s in order to receive 5 J/cm^2.

2.3. Biological assays

2.3.1. Cellular morphology. Qualitative changes in morphology were observed using an inverted light microscope and images were captured using an Olympus digital camera.

2.3.2. Cytokine analysis. The sandwich type optEIA™ enzyme-linked immunosorbent assay (ELISA) and flow cytometry were used to quantify cytokines IL-1β, IL-6 and TNF-α. Briefly, ELISA plates were coated by specific capture antibody overnight. It was then washed and blocked by assay diluents for inhibiting non-specific binding. Samples and standards were individually pipetted into wells and incubated at room temperature. Following the wash, cells were incubated with biotinylated anti-human monoclonal detection antibody, conjugated to streptavidin-horseradish peroxidase. Subsequent incubation with substrate was performed before the reaction was stopped and absorbance at a wavelength of 450 nm was read.

For flow cytometry, cytometric bead array (CBA) human flex sets were used. Supernatant from samples was mixed with capture beads and incubated in the dark. Thereafter samples were incubated with phycoerythrin (PE) detection reagent and incubated in the dark again. Samples were analyzed with the BD FACSArray bioanalyzer.

2.3.3. Apoptosis. Caspase-Glo 3/7 luminescence assay was used to quantify activated caspase-3 and -7 which play a vital role in the apoptosis pathway in mammalian cells. Briefly, cells were incubated and allowed to attach to the luminescent plate for 3 h. Then, Caspase-Glo 3/7 reagent was added followed by room temperature incubation. Luminescence was determined subsequently.

3. Results

3.1 Morphology

Cells were observed for any changes in morphology or reduction of wounded area after 1 or 24 h (1 h data not shown). The wounded area in irradiated normal wounded and diabetic wounded cells was decreased as compared to non-irradiated cells. Hypoxic cells had lost their characteristic morphology with some cells detaching. However, after irradiating cells had regained their characteristic morphology (Figure 1).

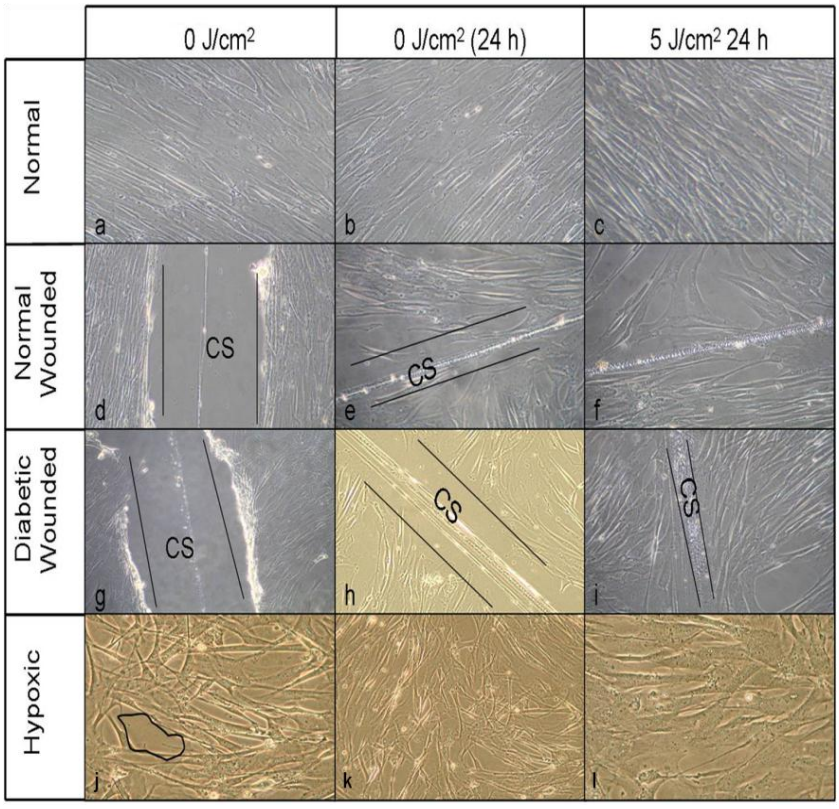

Figure 1. Morphological changes observed under the light microscope, in normal, normal wounded, diabetic wounded and hypoxic cells. Cells were exposed to 5 J/cm^2 at a wavelength of 830 nm and incubated for 24 h at 37 ºC. Migration was rapid in irradiated wounded cells although, in diabetic wounded cells they showed no contact in the central scratch (CS) as compared to normal wounded cells. Non-irradiated normal wounded cells had infiltrated and made contact at the CS, whereas diabetic wounded cells had infiltrated the CS. Spaces (encircled areas) in hypoxic cells were minimized post-irradiation and cells had regained their morphological features.

3.2. Cytokine analysis

3.2.1. *ELISA.* TNF-α was significantly decreased after 1 h incubation in irradiated normal, normal wounded ($P \leq 0.01$), diabetic wounded ($P \leq 0.05$) and hypoxic cells ($P \leq 0.01$) in comparison to their respective non-irradiated controls. After 24 h incubation the difference was seen in normal wounded, diabetic wounded ($P \leq 0.05$) and hypoxic cells ($P \leq 0.001$) as shown in Figure 2A. IL-1β had decreased significantly after 1 h incubation in irradiated normal and hypoxic cells as compared to their non-irradiated controls ($P \leq 0.05$). It was noted that after 24 h, IL-1β increased with an increase in severity of stress. Nonetheless, irradiated diabetic wounded and hypoxic cells showed significant IL-1β decrease ($P \leq 0.05$) as shown in Figure 2B. IL-6 was seen increasing with severity of the stress, excluding hypoxic stress; although the increase in irradiated normal, normal wounded and diabetic wounded cells was insignificant. However, a significant increase was seen in irradiated hypoxic cells after 1 h incubation. After 24 h incubation no significant results were observed (Figure 2C).

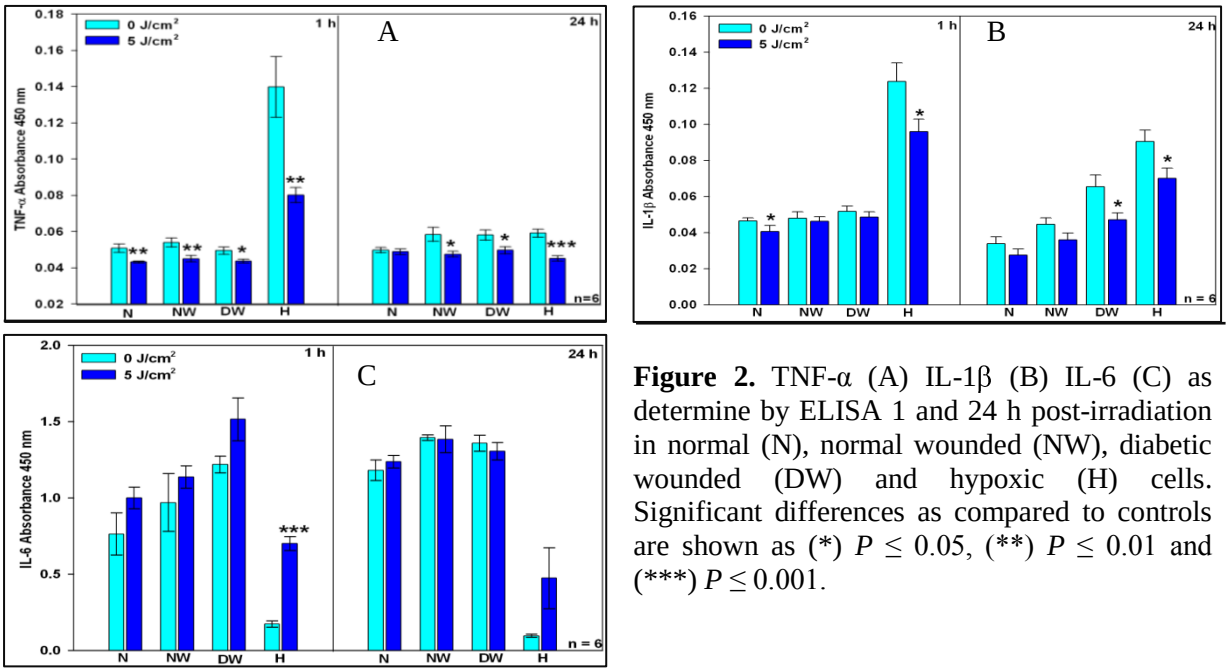

Figure 2. TNF-α (A) IL-1β (B) IL-6 (C) as determine by ELISA 1 and 24 h post-irradiation in normal (N), normal wounded (NW), diabetic wounded (DW) and hypoxic (H) cells. Significant differences as compared to controls are shown as (*) $P \leq 0.05$, (**) $P \leq 0.01$ and (***) $P \leq 0.001$.

3.2.2. *Flow cytometry.* TNF-α in cells irradiated at a wavelength of 830 nm did not produce any significant changes as compared to non-irradiated control cells, except in hypoxic cells ($P \leq 0.05$) after 1 h incubation. After incubation of cells for 24 h post-irradiation, only hypoxic cells had significantly decreased TNF-α compared to their non-irradiated control ($P < 0.01$) in Figure 3A.

IL-1β determination with flow cytometry showed no significant difference when normal, normal wounded, diabetic wounded and hypoxic cells were irradiated and compared to their non-irradiated controls after 1 h incubation. Despite irradiation with 5 J/cm^2 and an incubation of 24 h post-irradiation, there was no significant difference between irradiated and non-irradiated controls, except in diabetic wounded cells ($P \leq 0.01$) as shown in Figure 3B.

Irradiation seemed to increase expression of IL-6 although a significant increase was seen in irradiated normal and normal wounded cells after 1 h incubation ($P \leq 0.01$ and $P \leq 0.05$; respectively). However, IL-6 in both irradiated and non-irradiated hypoxic cells was decreased as compared to other stress models ($P \leq 0.001$). After 24 h incubation, irradiated normal wounded cells had a significantly decreased IL-6 ($P \leq 0.05$) and diabetic wounded cells that received irradiation had a significantly increased IL-6 ($P \leq 0.01$; Figure 3C).

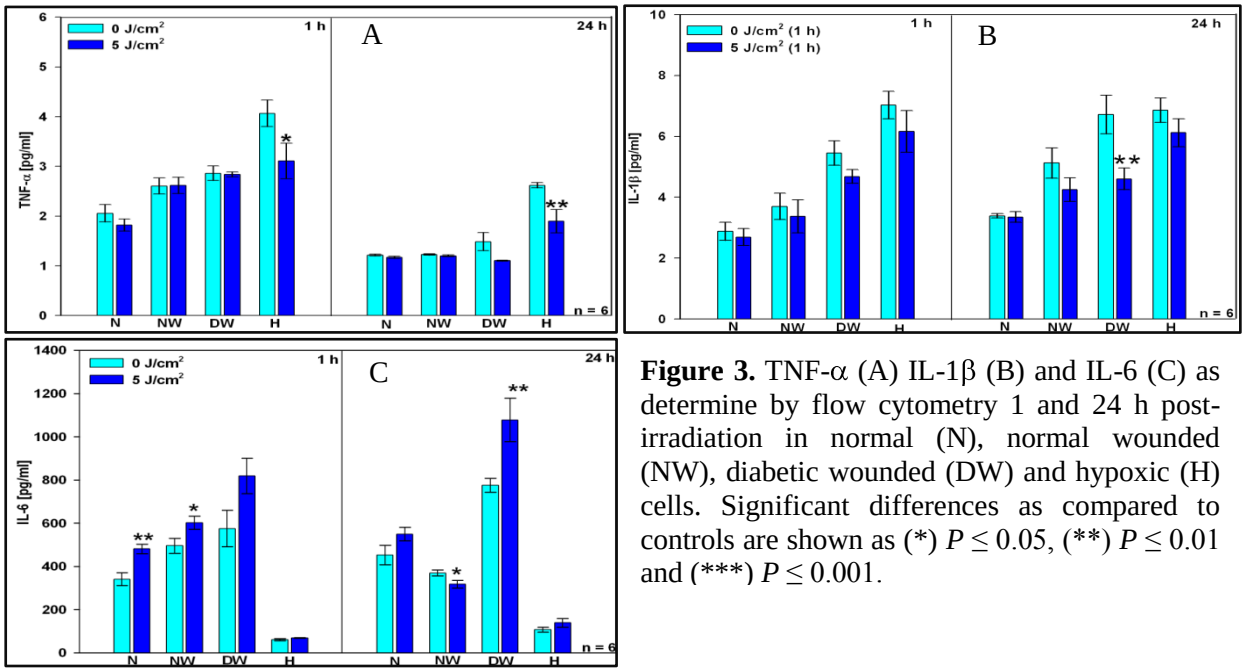

Figure 3. TNF-α (A) IL-1β (B) and IL-6 (C) as determine by flow cytometry 1 and 24 h post-irradiation in normal (N), normal wounded (NW), diabetic wounded (DW) and hypoxic (H) cells. Significant differences as compared to controls are shown as (*) $P \leq 0.05$, (**) $P \leq 0.01$ and (***) $P \leq 0.001$.

3.3. Apoptosis

Caspase 3/7, (Table 1), in irradiated cells was not significantly different as compared to their non-irradiated respective controls after 1 h incubation. Normal cells, despite irradiation, had a significantly decreased caspase 3/7 as compared to both irradiated and non-irradiated stressed models ($P \leq 0.001$). After 24 h incubation, a significant decrease was seen in irradiated normal wounded ($P \leq 0.001$) and diabetic wounded cells ($P \leq 0.01$). Comparison of the two incubation periods showed significantly decreased caspase 3/7 after 24 h incubation in all models ($P < 0.01$).

Table 1. Irradiated and non-irradiated cells were analyzed for caspase 3/7 activity after 1 or 24 h incubation. Significant differences as compared to non-irradiated controls are shown as (**) $P \leq 0.01$ and (***) $P \leq 0.001$.

Cells	0 J/cm² (1 h)	5 J/cm² (1 h)	0 J/cm² (24 h)	5 J/cm² (24 h)
Normal	1135.20 ±77.32[a]	1017.37 ±76.72	787.07 ±49.94	683 ±42.14
Normal Wounded	2235.74 ±132.43	2145.12 ±163.84	1699.73 ±108.41	932.75*** ±90.95
Diabetic Wounded	3345.81 ±91.07	3128 ±73.12	1814.97 ±92.90	1387.42** ±100.52
Hypoxic	3353.58 ±132.29	3117.97 ±63.28	2644.98 ±146.00	2432 ±111.28

[a]Standard Error

4. Discussion and Conclusion

LILI demonstrated effective wound healing as demonstrated by a reduction in the central scratch area and normalization of morphology of cells exposed to hypoxic conditions after treatment with a fluence of 5 J/cm^2. Hopkins *et al.*, [25] also observed similar findings; which were also a time-dependent

decrease in wound area. Ricci *et al*., [26] found hypoxic stressed endothelial cells regained their characteristic morphology post-irradiation. TNF-α and IL-1β are potent inducers of inflammation, therefore continuous or over-production of these pro-inflammatory cytokines may lead to delayed wound healing, cytotoxity or cell death. In hypoxic cells, these cytokines were found more elevated as compared to other stress models. Post-irradiation these cytokines were found decreased; suggesting that laser irradiation triggered other biological mediators that contribute to homeostasis or balancing of cellular physiology. Since diabetic wounds are difficult to heal or close effective, the decrease of these mediators found in irradiated diabetic wounded cells correspond with the reduced wounded area seen post-irradiation. IL-6 is a pleitropic cytokine which has both pro- and anti-inflammatory effects [27] and it is usually expressed in response to or together with IL-1 and TNF-α [28]. An increase seen in irradiated cells after 1 h incubation could suggest that IL-6 is radio-protective and thus plays an anti-inflammatory role since after 24 h incubation it is decreased in some irradiated models i.e. irradiated normal wounded cells (flow cytometry). Ali *et al*., [29] found increased IL-6 in hypoxic endothelial cells, whereas our hypoxic stressed cells exhibited diminished IL-6 expression. It is unclear why IL-6 is under-expressed in these cells, but it could be attributed to its pleiotropic nature as well as the ability to mediate the expression of IL-1β and TNF-α [30]. As it is difficult to rule which effects could have been exerted by the cytokines discussed above since their effects are also dependent on the pathway they follow, caspase 3/7 was determined to establish if cells were surviving or dying. Our findings suggested that laser irradiation had an anti-apoptotic effect on stressed cells. This also insinuates that the decrease in IL-1 and TNF-α was as a result of cells undergoing an anti-inflammatory state.

In conclusion, this study demonstrated the ability of LILI to be a potential therapeutic modality as it enhanced the recovery of stressed cells *in vitro*.

5. References

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