Irradiation at 830 nm Stimulates Nitric Oxide Production and Inhibits Pro-Inflammatory Cytokines in Diabetic Wounded Fibroblast Cells

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Background and Objective: Wound healing in diabetic patients remains a chief problem in the clinical setting and there is a strong need for the development of new, safe, reliable therapies. This study aimed to establish the effect of irradiating diabetic wounded fibroblast cells (WS1) in vitro on pro-inflammatory cytokines and the production of nitric oxide (NO).

Materials and Methods: Normal, wounded and diabetic wounded WS1 cells were exposed to an 830 nm laser with 5 J/cm^2 and incubated for a pre-determined amount of time. Changes in cellular viability, proliferation and apoptosis were evaluated by the Trypan blue assay, VisionBlue^{TM} fluorescence assay and caspase 3/7 activity respectively. Changes in cytokines (interleukin—IL-6, IL-1β and tumour necrosis factor-alpha, TNF-α) were determined by ELISA. NO was determined spectrophotometrically and reactive oxygen species (ROS) was evaluated by immunofluorescent staining.

Results: Diabetic wounded WS1 cells showed no significant change in viability, a significant increase in proliferation at 24 and 48 hours ($P<0.001$ and $P<0.01$ respectively) and a decrease in apoptosis 24 hours post-irradiation ($P<0.01$). TNF-α levels were significantly decreased at both 1 and 24 hours ($P<0.05$), while IL-1β was only decreased at 24 hours ($P<0.05$). There was no significant change in IL-6. There was an increase in ROS and NO ($P<0.01$) 15 minutes post-irradiation.

Conclusion: Results show that irradiation of diabetic wounded fibroblast cells at 830 nm with 5 J/cm^2 has a positive effect on wound healing in vitro. There was a decrease in pro-inflammatory cytokines (IL-1β and TNF-α) and irradiation stimulated the release of ROS and NO due to what appears to be direct photochemical processes. Lasers Surg. Med. 42:494–502, 2010.

Key words: IL-1β; IL-6; lasers; NO; ROS; TNF-α

INTRODUCTION

The process of wound healing is a highly co-ordinated process that involves a series of overlapping events controlled by a variety of cells, growth factors, cytokines and metabolic enzymes released at the wound site. Dysregulation of this co-ordinated event leads to impaired wound healing; an abnormality which is frequently seen in conditions such as diabetes. There are many causes of chronic wounds, with diabetes, pressure ulcers and venous stasis as the three most common causes [1]. Impaired wound healing is an incapacitating complication of diabetes often necessitating amputation and poses a serious challenge in clinical practice.

Growth factors and cytokines such as interleukin-1-beta (IL-1β), IL-6 and tumour necrosis factor-alpha (TNF-α) have diverse modes of action and are released during wound repair [2]. IL-1β and TNF-α are both well-known pro-inflammatory cytokines and have similar functions or effects; however, they do not share chemical or structural resemblance and their effects are interceded by specific receptors. Together with IL-1, TNF-α is the first cytokine known to be upregulated during the inflammatory phase of wound healing and contributes to the oxidative stress within the wound by generating reactive oxygen species (ROS) [3]. IL-6 is induced during acute phase reactions and usually expressed in response to or together with IL-1 and TNF-α [4]. However, contradictory effects have been reported [5]; it suppresses TNF-α, IL-1 and IL-12. Its vital role in wound healing is its ability to cause cell differentiation and proliferation. TNF-α is the most critical accelerator of diabetes [6].

ROS and reactive nitrogen species (RNS) act as molecular messengers during cell signalling; however, they have a biphasic effect, being both beneficial and detrimental depending on their concentration. ROS and RNS are generated during wound healing and are important mediators in this carefully controlled process, however in chronic wounds there is an uncontrolled production of these molecules. Nitric oxide (NO) is significantly reduced in chronic ulcers and impaired healing of diabetic wounds is

Contract grant sponsor: University of Johannesburg (UJ); Contract grant sponsor: National Research Foundation (NRF) of South Africa; Contract grant sponsor: Medical Research Council (MRC) of South Africa.
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Accepted 5 August 2009
Published online 15 July 2010 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/lsm.20812

thought to be related to this decrease [7,8]. Burrow et al. [9] demonstrated that normal skin fibroblasts produce more NO than diabetic human skin fibroblasts. Various studies show that phototherapy modulates NO both in vitro and in vivo [10–14].

Hyperglycemia is the key metabolic abnormality in diabetes mellitus that is believed to play the most prominent role in the development of diabetic complications [15]. A number of earlier studies showed that exposure of cells to hyperglycaemic conditions (20–40 mM), and thus mimicking uncontrolled diabetes, resulted in a restriction of cellular proliferation [16–19]. This restraint is more pronounced for higher glucose concentrations [16] and is expressed especially after protracted exposure to high glucose levels [20]. Previous studies have shown that continuous exposure of fibroblast cells to a glucose concentration of 22.6 mMol/L (17 mMol/L glucose added to media with a basal concentration of 5.6 mMol/L) slowed cellular migration and there was an increase in both cellular and DNA damage and apoptosis [21].

In cell culture, normal cells show contact inhibition of growth and population density stabilises at low levels; it is these properties that provide a suitable environment to study the cellular responses of cells as they react to an insult or injury [22]. The central scratch method is an in vitro wound model whereby the monolayer of cells are scratched down the centre of the culture flask. This method has been used with a variety of cells and, as the monolayers heal the wound in a characteristic manner, they have been used to study cell polarisation, matrix remodelling, cell migration and numerous other processes [23,24]. The injury model simulates in vivo mechanical trauma and the processes reflect the behaviour of individual cells as well as the properties of the cell sheet as a surrogate tissue [22]. The wounds heal in a stereotypical fashion with cells polarising toward the central scratch, initiate protrusion, migrate and close the wound. Progression of these events can be monitored by manually imaging samples at fixed time points [25–27].

The objective of this study was to determine if a wavelength of 830 nm at a dose of 5 J/cm^2 speeds up wound healing in wounded diabetic induced human skin fibroblast cells by increasing IL-6, ROS and NO and decreasing pro-inflammatory cytokines TNF-α and IL-1 β.

MATERIALS AND METHODS

Cell Culture

Human skin fibroblast cells were purchased from the American Type Culture Collection (WS1; ATCC CRL 1502; Adcock Ingram, Midrand, South Africa) and grown in complete Dulbecco's Modified Eagle's Medium as previously described by Hawkins and Abrahamse [28]. Normal, normal wounded and diabetic wounded cells were used in this study. An in vitro diabetic wound model was based on Rigau et al. [26], Hamuro et al. [29] and Vinck et al. [15]. Briefly, diabetic cells were continuously cultured in complete media containing an additional 17 mMol/L D-glucose. To determine the effects of the lasers, cells were detached by trypsinisation (1 ml/25 cm^2, 0.25% trypsin–0.03% ethylenediamine tetraacetic acid), and 6×10^5 cells in 3 ml complete culture media were seeded into 3.3-cm-diameter culture plates as determined by the Trypan blue exclusion test [28]. Plates were incubated overnight to allow cells to attach. A wound was induced 30 minutes before laser irradiation by scratching the cellular monolayer with a sterile 1 ml pipette [26].

Laser Set-Up and Irradiation

Cell cultures were chosen at random and WS1 cells were irradiated in the dark from the top with an 830 nm diode laser (power output 40 mW; spot size 9.1 cm^2; power density 4.4 mW/cm^2). Cells were irradiated once with a fluence of 5 J/cm^2, which was calculated at 18 minutes and 56 seconds. Unirradiated cells were treated in the same manner as irradiated cells, barring irradiation. Prior to irradiation, culture media was discarded and cells were rinsed with warm Hanks Balanced Salt Solution (HBSS), and replaced with 1 ml fresh media. Post-irradiation, cultures were incubated for a pre-determined amount of time (Table 1). Post-incubation cells were detached by trypsinisation and re-suspended in 500 µl culture media. All tests were performed on different populations ($n = 6$) of cells for each sample group and each biochemical assay was performed in duplicate.

Changes following laser irradiation were determined by measuring cellular viability (Trypan blue exclusion test), apoptosis (caspase 3/7 activity) and proliferation (VisionBlue^{TM} fluorescent assay). Cytokine expression was

TABLE 1. Study Design ($n = 6$)

	Proliferation	Apoptosis	Viability	NO	ROS	Cytokines (TNF-α; IL-1β; IL-6)
Incubation time	24 or 48 hours	1 or 24 hours	15 minutes, 1, 24 or 48 hours	15 minutes or 1 hour	15 minutes	1 or 24 hours
Method	Fluorescence	Caspase 3/7 activity	Trypan blue exclusion test	IF staining	Griess reagent system	ELISA
Data collection	Fluorescent spectroscopy	Luminescence	Light microscopy	Spectroscopy	Fluorescence microscopy	Spectroscopy

NO, nitric oxide; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-alpha; IL-1β, interleukin 1-beta; IL-6, interleukin-6; IF, immunofluorescent; ELISA, enzyme linked immunosorbent assay.

determined by ELISA, while ROS was determined by IF staining and NO by the Griess Reagent System.

Cellular Viability

The Trypan blue exclusion test was used to determine cellular viability in cells which had been incubated for 15 minutes, 1, 24 or 48 hours post-laser irradiation. An equal volume of 0.4% Trypan blue (Sigma-Aldrich, Johannesburg, South Africa, T8154) in HBSS was added to re-suspended cells and allowed to incubate at room temperature for 5–15 minutes. The number of viable (unstained) and non-viable (blue) cells were counted and the percentage viability calculated (number of viable cells divided by the number of total cells, multiplied by 100).

Apoptosis

The Caspase-Glo™ 3/7 assay (Whitehead Scientific, Johannesburg, South Africa, Promega, TB323) was used to measure the activity of caspase-3 and -7. The addition of reagent results in cellular lysis followed by substrate cleavage by caspase, and as a result, a luminescent signal is generated by luciferase. Negative controls consisted of reagent and culture media without cells. A positive control was included by inducing apoptosis in 1×10^6 cells/ml using 0.5 μg/ml Actinomycin D (Sigma-Aldrich, A5156-1VL). An equal volume of cells and reagent was added (25 μl), contents mixed and incubated at room temperature for 3 hours. Luminescence was read using the Victor-3 (Perkin-Elmer, Separation Scientific, Johannesburg, South Africa) and reported in reading light units (RLU).

Cellular Proliferation

Cellular proliferation of cells was determined using the VisionBlue™ Fluorescence Cell Viability Assay Kit (BiocomBiotech, Pretoria, South Africa, BioVision, K303-500), which provides a sensitive and easy means for quantifying cell proliferation. One hundred microlitres of cells was added to a 96-well microtitre plate and incubated at 37°C in 5% CO_2 for 2 hours to allow the cells to settle and attach. Following incubation, 10 μl (10% medium volume) VisionBlue™ reagent was added and plates incubated (37°C in 5% CO_2) for 2 hours. Fluorescence was then measured using the Victor-3 (Perkin-Elmer, Separation Scientific) at Ex/Em 560/595.

Cytokine Expression

The optEIA™ sandwich type enzyme-linked immunosorbent assay (ELISA) sets for human cytokine from BD Biosciences (Scientific Group, Johannesburg, South Africa) was used to determine IL-1β (BD 557953), TNF-α (BD 555212) and IL-6 (BD 555220). ELISA's were run according to the manufacturers' protocol. Briefly, each microwell plate was coated overnight at 4°C with specific capture antibody (1:250 in coating buffer). Plates were washed three times, blocked with assay diluent and incubated for 1 hour at room temperature. Plates were washed as before. Serial dilutions of standards were performed from the stock standard to generate a 9-point standard curve. One hundred microlitres of sample or standard was pipetted into their respective wells and incubated for 2 hours at room temperature. Plates were washed as before and incubated for 1 hour with working detector (biotinylated anti-human monoclonal detection antibody) conjugated to streptavidin-horseradish peroxidase. Plates were washed as before. Tetramethylbenzidine (TMB) substrate was added and plates were incubated for 30 minutes at room temperature in the dark. Stop solution was added and absorbance was determined at $A_{450\,nm}$ (Perkin-Elmer Victor 3).

Nitric Oxide

NO was determined spectrophotometrically using the Griess Reagent system (Whitehead Scientific, Promega, G2930). One means to investigate NO formation is to measure nitrite (NO_2), which is one of two primary, stable and non-volatile breakdown products of NO. This assay relies on a diazotisation reaction that was originally described by Griess in 1879 [30]. The Griess Reagent System uses sulphanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. A serial dilution of 100 μM nitrite was made in complete media (100–0 μM) and added to the respective wells of a 96-well plate. Equal amounts of culture media and sulphanilamide solution was added to the wells and incubated at room temperature, protected from light, for 10 minutes. Fifty microlitres of NED was added and cells incubated as before. Absorbance was measured at A_{540} nm (Perkin-Elmer, Victor-3).

Reactive Oxygen Species

ROS was determined in irradiated (5 J/cm^2) and control cells (0 J/cm^2) by immunofluorescent (IF) staining using the Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Scientific Group; Invitrogen, Molecular Probes, 136007). The assay is based on 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2 DCFDA), a reliable fluorogenic marker for ROS in live cells. In addition to carboxy-H_2DCFDA, the kit provides the common inducer of ROS production *tert*-butyl hydroperoxide (TBHP), as a positive control, and the blue-fluorescent cell-permeant nucleic acid stain Hoechst 33342. H_2DCFDA detects ROS such as hydrogen peroxide, singlet oxygen and hydroxyl radicals in living cells, but not superoxide anions or NOs [31]. Briefly, 6×10^5 cells were grown on heat sterilised coverslips in 3 ml complete culture media in a 3.3-cm-diameter culture plates. Post-laser irradiation, cells were washed with warm HBSS/Ca/Mg and labelled with 25 μM Carboxy-H_2DCFDA and incubated for 30 minutes at 37°C, protected from light. During the last 5 minutes of incubation, 1.0 μM Hoechst 33342 was added. Cells were washed and mounted using 0.1 M propyl gallate (Sigma-Aldrich, P3130) in glycerol/PBS (9:1). For the positive control, 100 μM TBHP was added to cells adhering to the coverslips and incubated (37°C in 5% CO_2) for 60 minutes. Fluorescence was viewed and images were taken with the Zeiss Live-Cell Imager.

Statistical Analysis

Experiments were repeated six times ($n = 6$). All assays were performed in duplicate and the mean was used. The results are represented as percentage change between irradiated cells (5 J/cm^2) and non-irradiated control cells (0 J/cm^2). Results were graphically presented and statistically analysed using Sigma Plot Version 8.0. A student t test and one-way ANOVA was performed to detect differences between the control and experiments, and as well as between experimental groups. Bonferroni correction was taken into account and all results remained significant ($P = 0.017$). Results were considered to be statistically significant when $P<0.05$. Statistical significance, compared to their respective control (0 J/cm^2) is shown in graphs as $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

RESULTS

Cellular Viability

Irradiation of WS1 cells at a wavelength of 830 nm with a fluence of 5 J/cm^2 did not have any significant effect on the viability of cells. Percentage viability was above 95% in all cell types.

Apoptosis

Post-irradiation, normal, normal wounded and diabetic wounded human skin fibroblast cells were incubated for 1 or 24 hours and caspase 3/7 activity was determined (Fig. 1a). There were no significant changes 1 hour post-irradiation, however, after 24 hours, there was a significant decrease in apoptosis of 82% and 31% in normal wounded ($P<0.001$) and diabetic wounded ($P<0.01$) cells respectively. Unirradiated normal cells showed a significant decrease in apoptosis at both 1 and 24 hours compared to unirradiated stressed cells ($P<0.001$), as did irradiated normal cells compared to irradiated normal wounded and diabetic wounded cells ($P<0.001$ at 1 hour and $P<0.05$ at 24 hours). Both unirradiated and irradiated diabetic wounded cells showed a significant increase in caspase 3/7 activity as compared to normal wounded cells ($P<0.001$) at both 1 and 24 hours. Caspase 3/7 activity significantly decreased in all irradiated cell types 24 hours post-incubation compared to 1 hour ($P<0.01$).

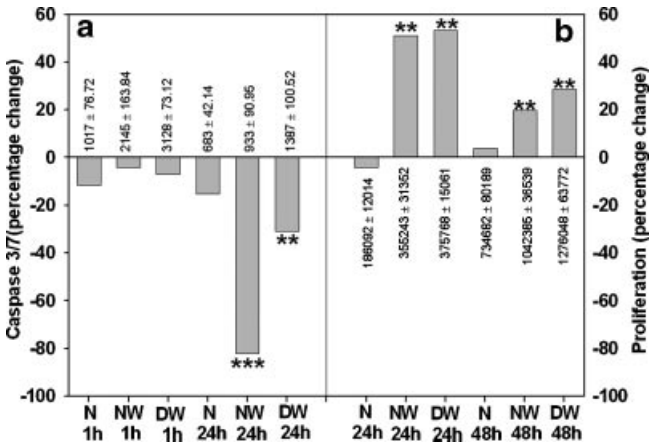

Fig. 1. Apoptosis (**a**) (luminescence, measured in relative light units—RLU) and proliferation (**b**) (measured in fluorescence, Ex/Em 560/595) was determined in normal (N), normal wounded (NW) and diabetic wounded (DW) cells irradiated at 5 J/cm^2 with 830 nm. Results are shown as percentage change between irradiated (5 J/cm^2) and non-irradiated control cells (0 J/cm^2) with the actual value and standard error written in. Significant differences are shown as $P<0.01$ (**) and $P<0.001$ (***). There was a significant decrease in caspase 3/7 activity 24 hours post-incubation in NW and DW cells. Note the significant increase in proliferation in NW and DW cells 24 and 48 hours post-incubation.

Cellular Proliferation

Cellular proliferation was determined in normal, normal wounded and diabetic wounded WS1 cells 24 or 48 hours post-irradiation at 830 nm with 5 J/cm^2 (Fig. 1b). There was an increase in proliferation of 51% and 19% in normal wounded cells irradiated for 24 or 48 hours respectively ($P<0.01$). Diabetic cells showed an increase of 53% and 28% respectively ($P<0.01$). Comparison of unirradiated cells showed an increase in proliferation in normal wounded and diabetic wounded cells 48 hours post-irradiation compared to normal cells ($P<0.05$). Comparison of irradiated cells showed an increase in normal wounded and diabetic wounded cells at both 1 and 24 hours ($P<0.01$) compared to normal cells. At 24 hours irradiated diabetic wounded cells showed an increase compared to irradiated normal wounded cells ($P<0.05$). All cells incubated at 37°C for 48 hours showed a significant increase in proliferation as compared to the same cells incubated for 24 hours ($P<0.001$).

Cytokine Expression

The optEIA^{TM} sandwich type ELISA sets was used to determine TNF-α, IL-1β and IL-6 in cells incubated for 1 or 24 hours post-irradiation. Normal, normal wounded and diabetic wounded cells incubated for 1 hour all showed a significant decrease in TNF-α by 18%, 20% and 13% respectively ($P<0.01$, $P<0.01$ and $P<0.05$ respectively) compared to non-irradiated controls (Table 2). At 24 hours, TNF-α levels returned to their natural levels in normal cells, however, levels were still significantly decreased in normal wounded and diabetic wounded cells ($P<0.05$) by 23% and 17% respectively. There was no significant difference between unirradiated cells or irradiated cells, except for the increase seen in unirradiated diabetic wounded cells at 24 hours ($P<0.05$) compared to unirradiated normal cells. The only difference seen between the two incubation times was in normal cells, with a decrease in TNF-α seen at 24 hours ($P<0.01$).

When IL-1β was determined in normal, normal wounded and diabetic wounded cells, normal cells irradiated at 830 nm with 5 J/cm^2 and incubated for 1 hour showed a significant decrease of 30% ($P<0.05$) compared to unirradiated control cells (Table 2), while diabetic wounded cells showed a significant decrease of 39%

TABLE 2. Interleukin-6 (IL-6), Tumour Necrosis Factor-Alpha (TNF-α) and Interleukin-1-Beta (IL-1β) Was Determined by ELISA in Cells Irradiated at 5 J/cm^2 With 830 nm and Incubated for 1 or 24 Hours

	1 hour		24 hours	
Cell	% Change	Absolute value	% Change	Absolute value
IL-6				
Normal	2.36	0.9992 ± 0.071	4.54	1.2365 ± 0.042
Normal wounded	14.78	1.1365 ± 0.073	−0.74	1.3834 ± 0.087
Diabetic wounded	19.6	1.5147 ± 0.141	−4.0	1.3052 ± 0.057
TNF-α				
Normal	−17.60**	0.0433 ± 0.005	−1.80	0.0489 ± 0.002
Normal wounded	−19.70**	0.0451 ± 0.002	−22.90*	0.0476 ± 0.002
Diabetic wounded	−13.00*	0.0438 ± 0.001	−16.70*	0.0498 ± 0.002
IL-1β				
Normal	−30.30*	0.0356 ± 0.004	−23.60	0.0275 ± 0.004
Normal wounded	−5.70	0.0453 ± 0.003	−23.50	0.0361 ± 0.004
Diabetic wounded	−6.60	0.0486 ± 0.003	−38.80*	0.0472 ± 0.004

Results are shown as percentage change between irradiated (5 J/cm^2) and non-irradiated control cells (0 J/cm^2), with the absolute value and standard error included. Significant differences are shown as $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). After 1 hour incubation, there was a significant decrease in TNF-α in all cells, which remained significantly decreased in NW and DW cells 24 hours later. There was a significant decrease in IL-1β in N cells 1 hour post-irradiation and in DW cells 24 hours post-irradiation.
± Standard error.

24 hours post-irradiation ($P<0.05$). The decreases seen in the other cell types were insignificant. Unirradiated diabetic wounded cells showed a significant increase in IL-1β at 24 hours compared to unirradiated normal and unirradiated normal wounded cells ($P<0.01$ and $P<0.05$ respectively). Comparison of irradiated cell types showed a significant increase at both 1 and 24 hours in diabetic wounded cells compared to normal cells ($P<0.05$ and $P<0.01$ respectively). The only significant difference seen between the two incubation times was in unirradiated normal cells, with a decrease seen at 24 hours ($P<0.05$).

When normal, normal wounded and diabetic wounded WS1 cells were irradiated once at 830 nm with 5 J/cm^2 and incubated for 1 or 24 hours, there was no significant change in IL-6 levels in irradiated cells compared to unirradiated controls (Table 2). Comparison of unirradiated cell types showed a significant increase in IL-6 in diabetic wounded cells at both 1 and 24 hours ($P<0.05$ and $P<0.01$ respectively). Unirradiated normal wounded cells only showed an increase at 24 hours ($P<0.05$). Irradiated diabetic wounded cells showed a significant increase at 1 hour compared to both irradiated normal and normal wounded cells ($P<0.01$ and $P<0.05$ respectively). Cells which were incubated for 24 hours showed an increase in IL-6 compared to cells incubated for 1 hour, with significances seen in unirradiated and irradiated normal cells ($P<0.05$) and irradiated normal wounded cells ($P<0.05$).

Nitric Oxide

Normal, normal wounded and diabetic wounded human fibroblast cells were irradiated at 830 nm with 5 J/cm^2 and incubated for 15 minutes or 1 hour at 37°C. NO was determined at A_{540} nm. Fifteen minutes post-irradiation, all cells showed a significant increase in NO ($P<0.01$), (Fig. 2). Normal and diabetic wounded cells showed an increase of 49%, while normal wounded cells showed an increase of 45%. This increase was no longer evident at

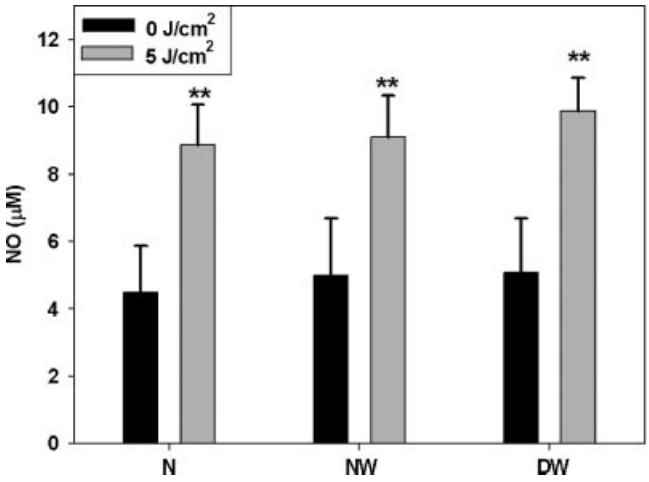

Fig. 2. Nitric oxide (NO) was determined by the Griess reagent system in normal (N), normal wounded (NW) and diabetic wounded (DW) cells irradiated at 5 J/cm^2 with 830 nm and incubated for 15 minutes. There was a significant increase in NO in all cells incubated for 15 minutes. Significant differences are shown as $P<0.01$ (**).

1 hour. Unirradiated cells showed no significant difference between the incubation times, while irradiated normal, normal wounded and diabetic wounded cells showed a significant decrease 1 hour post-irradiation compared to cells incubated for 15 minutes ($P<0.001$, $P<0.05$ and $P<0.01$ respectively).

Reactive Oxygen Species

ROS was determined 15 minutes post-laser irradiation in unirradiated and irradiated normal and diabetic cells (Fig. 3) by fluorescent staining. Post-irradiation, both normal and diabetic cells showed more green fluorescence than unirradiated normal and diabetic cells respectively. Irrespective of irradiation, diabetic cells showed more ROS than normal cells.

DISCUSSION

The development of new therapies for wound healing requires an understanding of the mechanisms involved, including underlying disease conditions, and translating these mechanisms into useful agents. Diabetes is known to be associated with poor wound healing and is responsible for 50–70% of all non-traumatic amputations and it is estimated that 15% of all diabetic patients will develop an ulcer on the feet or ankles at some time during the disease course [32]. Diabetic wounds are predominantly

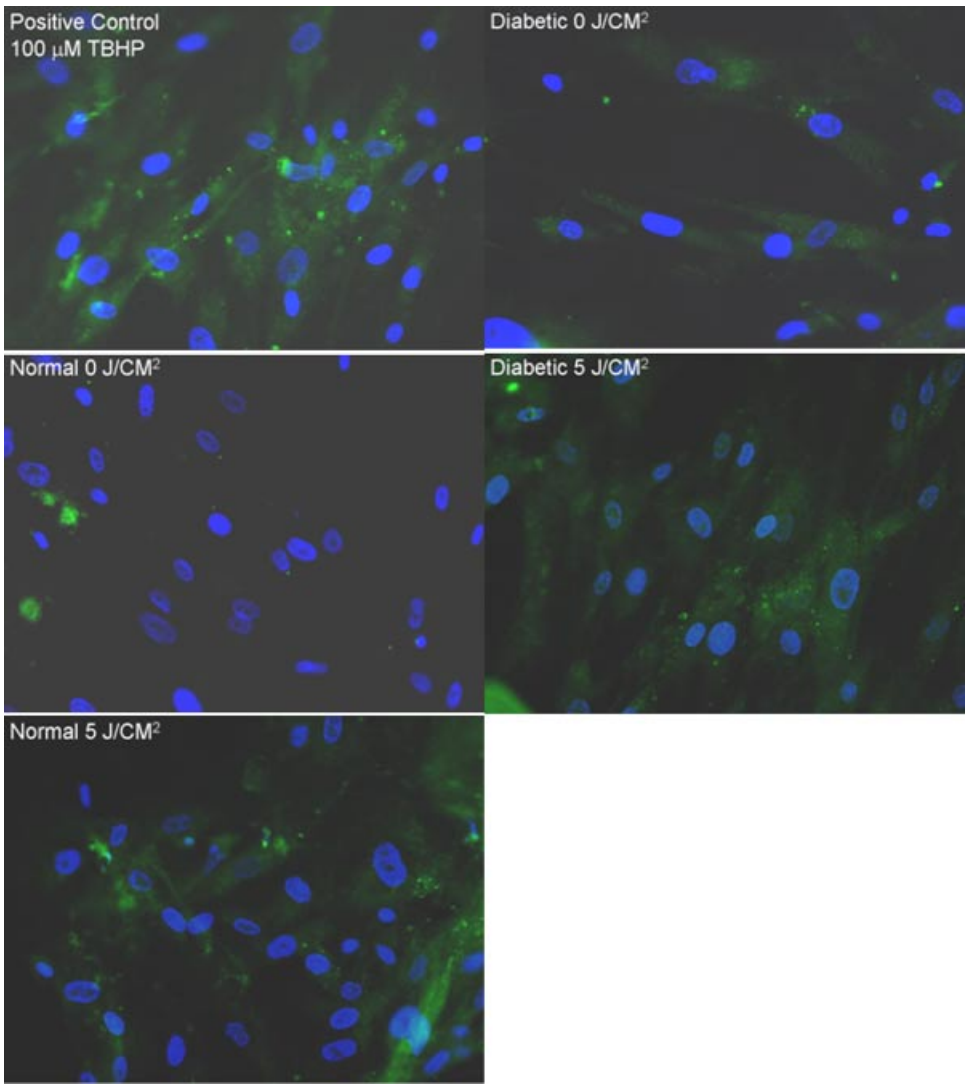

Fig. 3. Reactive oxygen species (ROS) was determined by fluorescent microscopy in non-irradiated (0 J/cm^2) and irradiated (5 J/cm^2) normal and diabetic cells. A positive control (100 μM *tert*-butyl hydroperoxide (TBHP)) was included. ROS fluoresced green, while the nuclei fluoresced blue. Little ROS is seen in non-irradiated cells, while irradiated cells show and abundance of ROS. Diabetic cells show more green fluorescence than normal cells. [Figure can be viewed in color online via www.interscience.wiley.com.]

characterised by peripheral neuropathy, structural deformity, altered immune function or increased susceptibility to infection, decreased wound NO production, and often hypoxia/ischemia [33,34]. Treatment of diabetic wounds includes debridement, mechanical load relief, topical antibiotics and dressings, while newer developments include the use of bioengineered skin equivalents, growth-factor therapy and hyperbaric oxygen treatment. A number of studies have shown that laser irradiation, using appropriate parameters, is beneficial to a wide range of conditions, including wound healing in diabetic patients.

Al-Watban et al. [35] found a wavelength of 633 nm with a dose of 10 J/cm^2 to be the most beneficial in treating wounds in diabetic mice. Maiya et al. [36] also found laser therapy to be beneficial in hastening the healing process in diabetic rats (632.8 nm with 4.8 J/cm^2), while Rabelo et al. [37] found a dose of 10 J/cm^2 beneficial. Al-Watban [38] suggests that 633 nm laser therapy should be given three times per week at 4.71 J/cm^2 per dose for diabetic burns, and three times per week at 2.35 J/cm^2 per dose for diabetic wound healing as actual doses for human clinical trials.

Inflammatory cytokines such as IL-1β and TNF-α have also been shown to be increased in non-healing wounds, as well as in diabetic patients [39]. In addition, TNF-α is autostimulative and can induce the secretion of IL-1β, resulting in a persisting cycle of inflammation [40]. TNF-α is responsible for apoptosis by binding to its receptor, TNFRI which contains a death domain, and activating the caspase cascade. IL-1β enhances TNF-α induced apoptosis [41] and is capable of inducing other cytokines such as TNF, IL-6, IL-2 and apoptosis which can be mediated via NO production by increasing secretion of inducible nitric oxide synthase (iNOS). IL-6 exerts a variety of effects on cells and is involved in immune activity, the acute-phase response to injury and infection, inflammation, oncogenesis and hematopoiesis, as well as exerting growth-inducing, growth-inhibitory and differentiation-induction effects [42–45]. Aimbire et al. [46] found that irradiation of rats with an immune-complex induced lung injury at 650 nm with 0.11 J reduced TNF-α concentration in bronchoalveolar lavage fluid. Mafra de Lima et al. [47] and Boschi et al. [48] also found a reduction in TNF-α post-irradiation, while Safavi et al. [49] found a significant decrease in the gene expression of IL-1β and no significant difference in TNF-α in irradiated rats. Shimizu et al. [50] also showed that laser irradiations caused the inhibition of IL-1β and Boschi et al. [48] found a decrease in NO and IL-6.

There was no significant difference in viability, proliferation, IL-6, TNF-α, IL-1β and NO between unirradiated normal wounded and unirradiated diabetic wounded cells at both 1 and 24 hours, thus any differences seen in irradiated diabetic wounded cells was a direct result of laser irradiation. There was an increase in caspase 3/7 activity in diabetic cells, this corresponds with previous studies [21] and work conducted by Susztak et al. [51].

Irradiation at a wavelength of 830 nm with a fluence of 5 J/cm^2 stimulated cell survival. There was no negative effect on cellular viability in irradiated cells, thus laser irradiation did not induce additional damage on cells. In fact it protected cells from apoptosis as seen by the decrease in caspase 3/7 activity. This study showed a significant increase in proliferation in both normal wounded and diabetic wounded cells in vitro at 24 and 48 hours. The increase was more pronounced 24 hours post-irradiation, with increases of 50%. Gavish et al. [52] also found an increase in proliferation in porcine aortic smooth muscle cells irradiated at 780 nm with 2 J/cm^2. However, they found a greater increase at 24 hours as opposed to 48 hours. Hawkins and Abrahamse [28] found an increase in proliferation in wounded fibroblast cells (WS1) irradiated with a He–Ne laser with 5 J/cm^2, while Houreld and Abrahamse [53] found an increase in diabetic wounded WS1 cells irradiated at 830 nm with 5 J/cm^2.

Irradiation of normal wounded and diabetic wounded cells at 830 nm with 5 J/cm^2 had an anti-inflammatory effect on cells, with decreases in TNF-α seen in normal, normal wounded and diabetic wounded cells 1 hour post-irradiation, and decreases in normal wounded and diabetic wounded cells 24 hours post-irradiation. A decrease in IL-1β was seen in normal cells 1 hour post-irradiation and in diabetic wounded cells 24 hours post-irradiation. These decreases in pro-inflammatory cytokines corresponds with other studies [46–50,54]. There was no TNF-α induced apoptosis in cells as seen by the decrease in TNF-α and caspase 3/7 activity and an increase in proliferation. Cells were stimulated to enter the cell survival pathway.

Several papers on laser irradiation have shown significant increases in IL-6 [42,55,56], this study showed an insignificant increase at 1 and 24 hours ($P = 0.08$ and $P = 0.514$ respectively). IL-6 has been linked to the pathogenesis of type 1 diabetes [43,57,58] and altered IL-6 levels have also been associated with delayed wound healing in diabetes [59]. This study showed that although there was an initial insignificant increase in IL-6, levels decreased and there was no negative effect on wound healing in vitro.

At a molecular level, the effects of laser therapy remain illusive. Many studies have suggested that these effects are as a result of ROS which then participate in various redox reactions. Eichler et al. [11] found that both red and infrared light stimulated the production of ROS in rat cardiocytes. Lindgård et al. [13] demonstrated that irradiation at 634 nm (35.7 W/cm^2) could stimulate the release of NO in human monocytes within 20 minutes and that the release was not coupled to the activation of iNOS or endothelial NOS (eNOS). They also demonstrated the intracellular release of ROS. Pal et al. [31] irradiated normal human fibroblasts with a He–Ne laser (0.5–16 J/cm^2; 0.64–1.16 mW/cm^2) and found that the kinetics of ROS generation was strongly dependent on laser fluence rather than laser intensity.

In this study, all cell types showed an increase in NO 15 minutes post-irradiation. There were no significant changes at 1 hour, and the decrease at 1 hour was significant compared to 15 minutes. In this study ROS production was determined by immunofluorescence staining. WS1 cells irradiated with 5 J/cm^2 showed more fluorescence than unirradiated cells. As expected, diabetic cells showed more ROS than normal cells. TNF-α is capable

of activating NF-κB which then translocates from the cytosol to the nucleus where it initiates the production of ROS. In this study it appears as though the increase in ROS and NO is directly released due to a photochemical process since the increases were seen 15 minutes post-irradiation. It appears plausible that TNF-α could not have stimulated ROS production (via NF-κB) since TNF-α levels were decreased 1 hour post-irradiation.

This study shows that laser therapy might prove beneficial for wound healing, including healing of diabetic wounds. Irradiation of wounded diabetic cells in vitro at a wavelength of 830 nm using 5 J/cm^2 did not induce additional damage, significantly increased proliferation, ROS and NO production and significantly decreased pro-inflammatory cytokines TNF-α and IL-1β and caspase 3/7 activity. Irradiation of normal and diabetic induced WS1 cells stimulated the release of intracellular ROS and NO due to what appears to be direct photochemical processes.

ACKNOWLEDGMENTS

All lasers were supplied and set-up by the National Laser Centre (NLC) of South Africa.

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