

Photobiomodulation at 660 nm Modifies Apoptosis Regulators Bcl-2 and Caspase-3 in Diabetic Wounded Cell Models *in Vitro*

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Abstract. While it has been identified that photobiomodulation (PBM) improves hyperglycaemic impaired cutaneous wound healing, there is insufficient knowledge about the cellular mechanism involved. During the normal wound healing process, apoptosis is fundamental for the elimination of inflammatory cells and scar formation. Under diabetic conditions there is increased apoptosis, making the wound susceptible to infection and delayed healing due to a persistent inflammatory state. This study examined the assumption that PBM at 660 nm modifies apoptosis regulators Bcl-2 and caspase 3 in diabetic wounded cell models *in vitro*. WS1 skin fibroblasts were modelled into normal (N), wounded (W), and diabetic wounded (DW), and were irradiated at a wavelength of 660 nm and a fluence of 5 J/cm². Non-irradiated cells (0 J/cm²) were employed as controls. Following 48 h of incubation, cells were evaluated for morphology, percentage migration rate, wound closure, viability (trypan blue and MTT), and apoptosis (Caspase 3/7, Bcl-2, and Annexin V/PI). PBM at 660 nm significantly increased cell migration, wound closure and viability, and reduced apoptosis in DW cells. PBM at 660 nm and 5 J/cm² increases cell viability and *in vitro* diabetic wound healing through the reduction of apoptosis by diminishing caspase activity and increasing the release of Bcl-2. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: fibroblasts; photobiomodulation; wound; apoptosis; Bcl-2; caspase; diabetes mellitus; laser therapy.

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1 Introduction

The wound healing process requires the combination of complicated molecular and cellular events that take place in succeeding phases of inflammation, formation of granulation tissue, and remodelling. In the first phase, inflammatory cells including neutrophils infiltrate the wound to eliminate debris and avoid infection [1]. The inflammatory phase is followed by the formation of granulation tissue, and is characterised by fibroblast and endothelial cell proliferation. Regulated by inflammatory factors, fibroblast cells migrate to the fibrin clot, proliferate and facilitate angiogenesis and the

construction of granulation tissue. The presence of fibroblast cells supports migration of other cells involved in the healing process by producing matrix metalloproteinases (MMPs) and extracellular matrix (ECM) proteins. Towards the beginning of the remodelling phase, fibroblast cells, stimulated by C-X-C Motif Chemokine Ligand 8 (CXCL8) and transforming growth factor- β (TGF- β), differentiate into myofibroblasts, critical cells involved in the remodelling phase [2, 3]. This phase mainly involves structural adjustment of the deposited collagen and development of the stretchy and tensile strength of the wound. Thus, fibroblast cell viability, proliferation, and migration is

critical in a standard cutaneous wound healing process [1].

In diabetes mellitus (DM), advanced glycation end products (AGEs) trigger apoptosis due to the increased presence of reactive oxygen species (ROS). AGEs are formed as a consequence of glucose-protein concentration, which when increased affect wound healing and remodelling [4, 5]. Cellular caspases are a frequently activated death protease, catalysing the precise cleavage of several key intracellular proteins [6, 7]. The mitochondrial or intrinsic pathway for apoptosis is triggered by a diversity of inducements including hypoxia and oxidative stress, resulting in mitochondrial outer membrane permeabilisation and discharge of apoptogenic cytochrome c into the cytoplasm to form an apoptosomal complex which activates the effector caspases -3 and -7 to induce apoptosis [8]. On the other extreme, B cell lymphoma 2 (Bcl-2), the most distinctive anti-apoptotic protein, obstructs apoptosis by creating a heterodimer with BAX (Bcl-2-like protein 4), thereby controlling the concentration and antioxidant effect of ionized calcium, and the activities of caspase-3 and -7 [9].

DM is a systemic condition resulting from insulin deficiency or loss of responsiveness in its target tissue, resulting in hyperglycaemia [5]. It impairs the wound healing process including defects in the inflammatory response, and raised fibroblast cell apoptosis. Literature shows that the mechanism of diminished diabetic wound healing is related to increased apoptosis and reduced viability of fibroblast cells [10, 11]. According to the 2021 International Diabetes Federation (IDF) Diabetes Atlas [12], approximately 540 million adults aged between 20 and 79 years are living with DM, and this number is expected to rise to approximately 645 million before 2030 and 784 million before 2045. Patients with DM have a 25% lifetime risk for developing a chronic diabetic wound of the foot, and approximately between 14% and 24% of these patients may require a major or minor non-traumatic limb amputation, mostly due to acute gangrene [13].

There are several treatment methods for diabetic wounds with each method having its own efficacy short falls. Photobiomodulation (PBM) is a new treatment method used for the treatment of diabetic wounds, and its benefits have been demonstrated to be significant in reducing acute pain, amendment of tissue scars, and improving cellular proliferation and generation of viable cells [14, 15]. Mitochondria are the basic site for the primary effects of PBM, leading to raised production of adenosine triphosphate (ATP), alteration of ROS, and stimulation of transcription factors, leading to raised fibroblast cell proliferation, viability, and migration [16]. In the mitochondria, photons are absorbed by components of the respiratory chain, mainly complex IV or cytochrome c oxidase (COX), which moves electrons to form molecular oxygen [17].

PBM therapy is known to regulate healing activities in diabetic wounds. However, the mechanism by which these activities are affected is not completely understood.

This investigation examined the assumption that 660 nm PBM reduces apoptosis by increasing the release of Bcl-2 and reducing caspase activity in diabetic wounded fibroblast cells *in vitro* to gain new insights into mechanisms by which PBM induces wound healing in DM.

2 Materials and Methods

American Type Culture Collection WS1 human skin fibroblast cells (ATCC®, CRL-1502™) were cultured using standard culture procedures. Three models were used, namely normal (N), wounded (W), and diabetic wounded (DW). An *in vitro* diabetic cell model was realised by uninterruptedly cultivating WS1 cells in minimum essential medium eagle (MEM) with a basal glucose concentration of 5.6 mM, to which an extra 17 mM D-glucose was added, giving a final glucose concentration of 22.6 mM, thus mimicking a hyperglycaemic condition [18]. Cells (6×10^5) were seeded in 3.4 cm diameter tissue culture plates and incubated for 24 h at 37 °C in 5% CO₂ for attachment, after which a wounded cell model was created using the central scratch assay performed on a confluent monolayer and incubated for 30 min pre-irradiation. A central scratch assay creates a cell free zone bordered by cells on both sides of the “wound” [19]. After 30 min incubation, cell culture plates, with the lids off, were subjected to laser light from above in the dark and incubated for 48 h before detachment using 300 µL of TrypLE™ Select (Life Technologies, Gibco, 12563-029). All cell models were analysed for cell morphology, and the percentage migration rate and wound closure in wounded cell models (W and DW) using an inverted light microscope and the analySIS getIT software. All cell models were assessed for viability using the Trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays, and apoptosis using the Caspase-Glo® 3/7 assay for caspase activity, enzyme linked immunosorbent assay (ELISA) for the release of Bcl-2, and Annexin V/PI-fluorescein isothiocyanate (FITC) and propidium iodide (PI) to categorise membrane phosphatidyl serine in apoptotic cells and membrane damage in necrotic cells. Table 1 displays the laser parameters utilised in this study. Non-irradiated cells were used as controls. All experiments were analysed in duplicate, and were repeated three times ($n = 3$).

2.1 Cell Morphology and the Percentage Migration Rate

In this study, the percentage migration rate and wound closure were analysed by measuring the distance between the borders of the “wound” at 3 specific points marked on the culture dishes and using the graduated locator marks on the mechanical stage for easy return to the area for observation. The results were calculated as the percentage migration rate per point using the following Eq.:

$$(A_{0h} - A_{time})/A_{0h} \times 100,$$

where A_{0h} is the distance between the borders of the “wound” at 0 h, and A_{time} is the distance between the borders of the “wound” at 48 h [20–22]. The average rate of the points was used to get the average percentage of the 3 repeats ($n = 3$).

Table 1 Laser parameters.

Light source	Diode laser
Wavelength (nm)	660
Emission	Continuous wave
Power output (mW)	101
Power density (mW/cm ²)	11
Spot size (cm ²)	9.1
Energy density (J/cm ²)	5
Irradiation time	7 min 35 s
Energy (J)	46

2.2 Cell Viability

2.2.1 Trypan Blue

To establish the number of viable and healthy cells present in each cell model, the Trypan blue (Sigma-Aldrich, T8154) exclusion assay was used. Cells were detached using TrypLE™ Select, suspended in 1 mL pre-warmed Hank’s balanced salt solution (HBSS; Sigma-Aldrich, 55037c), and 10 µL of the suspension was mixed to an equal volume (10 µL) of 0.4% Trypan blue stain. The mixture was gradually mixed and left to incubate for approximately 3 min, after which 10 µL of the mixture was loaded into the Invitrogen Countess® II FL disposable counting slide chambers and loaded into the Invitrogen Countess® II FL automated bench-top cell counter for analysis.

2.2.2 The 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

The MTT colorimetric assay (Roche, 11465007001) was used to confirm cell viability. In this assay, tetrazolium salts are cleaved to formazan by the succinate-tetrazolium reductase system belonging to the respiratory chain of the mitochondria, and is only functional in metabolically undamaged viable cells. Cells (5×10^4) were resuspended in 100 µL media/well of a 96-well tissue culture plate and incubated for 4 h at 37°C in 5% CO₂ for attachment, after which, 10 µL MTT reagent was added and incubated at 37 °C in 5% CO₂ for 4 h. Following this, a 100 µL of the solubilisation buffer was added, and the plate was incubated overnight at 37°C in 5% CO₂. The plate was then read using the VICTOR Nivo® Multimode Plate Reader (PerkinElmer) at 570 nm.

2.3 Apoptosis

2.3.1 The Caspase-Glo® 3/7 Assay

The Caspase-Glo® 3/7 assay (Promega, AnaTech, G8090) is centred on measuring standardised luminescent activity of the members of the cysteine aspartic acid-specific protease (caspase) family, caspase-3 and -7, that play an essential effector role in cell apoptosis. In this assay, addition of the Caspase-Glo 3/7® reagent causes cell lysis, with subsequent caspase cleavage of a tetra peptide sequence DEVD substrate, and the production of luminescence. Approximately 2×10^4 cells were suspended in a total volume of 100 µL media/well of a white-walled 96-well plate and incubated for 4 h at 37 °C in 5% CO₂ for attachment. After incubation, 100 µL of Caspase-Glo® 3/7 reagent was added, and using an orbital shaker, the contents were gently mixed at 300–500 rpm for 30 s and incubated at room temperature for 1 h. The luminescence of each sample was read in the VICTOR Nivo® Multimode Plate Reader (PerkinElmer). The emitted luminescence is proportional to the extent of caspase-3 and -7 activity in the cells.

2.3.2 The Enzyme-Linked Immunosorbent Assay (ELISA)

The sandwich ELISA was used to quantitatively evaluate the concentration of the released Bcl-2 (Human Bcl-2 ELISA Kit, Abcam, ab202411) in culture media according to the manufactures’ guidelines. Culture media was collected 48 h post-irradiation, frozen, and stored for further analysis. All three repeats were run together on the same plate. Briefly, 100 µL of standard or sample was dispensed in duplicate to a 96-well plate coated with immobilised human Bcl-2 capture antibody. Wells were washed 4× with wash buffer (Bio-Rad, PW40 microplate washer), followed by the addition of 100 µL of biotinylated anti-human Bcl-2 antibody. Unbound biotinylated antibody was washed and 100 µL of HRP-conjugated streptavidin added. After another washing step, 100 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added, and incubated at room temperature in the dark on a shaker (moderate shaking) for 30 min. The reaction was stopped through the addition of 50 µL stop solution, and absorbance measured at 450 nm by means of the VICTOR Nivo® Multimode Plate Reader (PerkinElmer). The developed colour was relative to the concentration of the bound Bcl-2.

2.3.3 The Annexin V/PI–Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI) Assay

The Annexin V/PI–fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BD Biosciences, 556547, The Scientific Group, South Africa) flow cytometry assay is mostly used to categorise membrane phosphatidyl serine in apoptotic cells and destruction of the membrane in necrotic cells. The assay was performed on the BD Accuri™ C6 flow cytometer matching the manufacturers’

instructions. In brief, following treatment and incubation, cells from all models were detached using TrypLE™ Select and washed 3× in HBSS. Cells were resuspended in 1× binding buffer at a concentration of 10^6 cells/mL. A 100 µL of the cell suspension was stained with 5 µL each of the Annexin V–FITC and PI reagents for 10 min in the dark at room temperature. Flow cytometric analysis at a rate of 400 events per second with a limit of 350 µL was performed within 1 h. Annexin V–FITC was detected as green fluorescence, and PI as red.

2.4 Statistical Analysis

SigmaPlot version 14 (Systat Software, Inc.) was used for statistical analysis, and the statistical difference between groups was distinguished by using the Student's *t*-test. The one way analysis of variance (ANOVA) followed by Dunnett's test was used to compare the differences between the cell models. A statistically significant difference between two groups determined using a *p*-value does not show the true magnitude of the difference which can only be determined using an effect size. To quantify the effect size between unirradiated and irradiated groups, Cohen's *d* was employed. Results are shown as standard error of the mean (SEM) and statistical significance is shown in the graphs as **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

3 Results

3.1 Cell Morphology and Migration Rate

There was no cellular morphological difference in both irradiated and non-irradiated normal and diabetic wounded cell models 48 h following irradiation. The cells appeared aligned, mostly in parallel, slender, and

stretching outwards, with protrusions, proliferating and spreading to form a single stratum of cells (Fig. 1).

This study assessed the distance between the borders of the “wound” (Fig. 2) which were used to calculate the percentage migration rate. A significant percentage migration rate was observed in irradiated W and DW cells with large effect sizes (Cohen's *d* of 3.027 and 2.375, respectively) as compared to their unirradiated controls (Table 2). When compared to unirradiated (0 J/cm²) W cells, a significant decline in the percentage migration rate was noted in unirradiated (0 J/cm²) DW cell models (*p* < 0.01). However, irradiated (5 J/cm²) DW cells displayed no difference in the percentage migration rate as compared to irradiated (0 J/cm²) W cells (*p* = 0.842), and as shown in Fig. 2, there was partial and complete wound closure observed in irradiated (5 J/cm²) W and DW cell models, respectively, 48 h after irradiation.

3.2 Cell Viability

Cellular viability represents the percentage of viable/live cells of the culture. The Trypan blue exclusion and MTT assays were used to assess for cell viability in all models. Using the Trypan blue exclusion assay (Fig. 3), a significant increase in cellular viability was observed in irradiated (5 J/cm²) W and DW cells with large effect sizes (Cohen's *d* of 3.377 and 4.859, respectively). Using one-way ANOVA, there was a significant decline in percentage cell viability in unirradiated (0 J/cm²) W cells (*p* < 0.05) and DW cells (*p* < 0.001) when compared to unirradiated (0 J/cm²) N cells. When cells were irradiated, this difference was no longer evident in irradiated (5 J/cm²) W cells as compared to unirradiated (0 J/cm²) N cells.

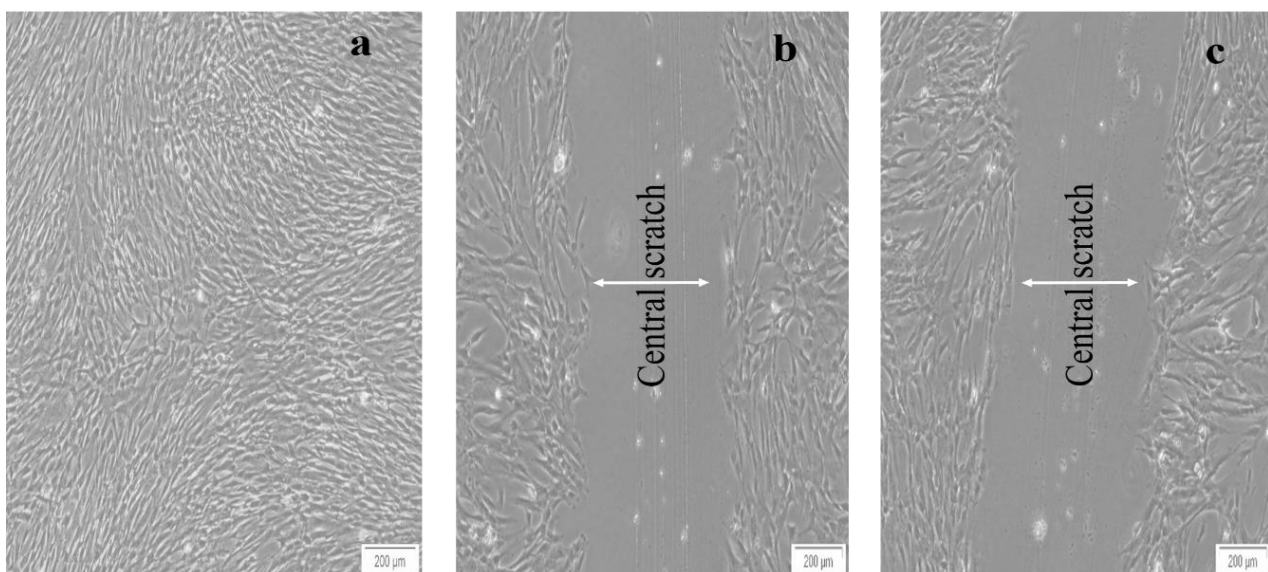


Fig. 1 Representative micrographs showing cellular morphology observed in (a) normal (N), (b) wounded (W), and (c) diabetic wounded (DW) WS1 human skin fibroblast cells. Wounded cell models were created using the central scratch assay performed on a confluent monolayer, and is highlighted by a white line. Magnification ×200.

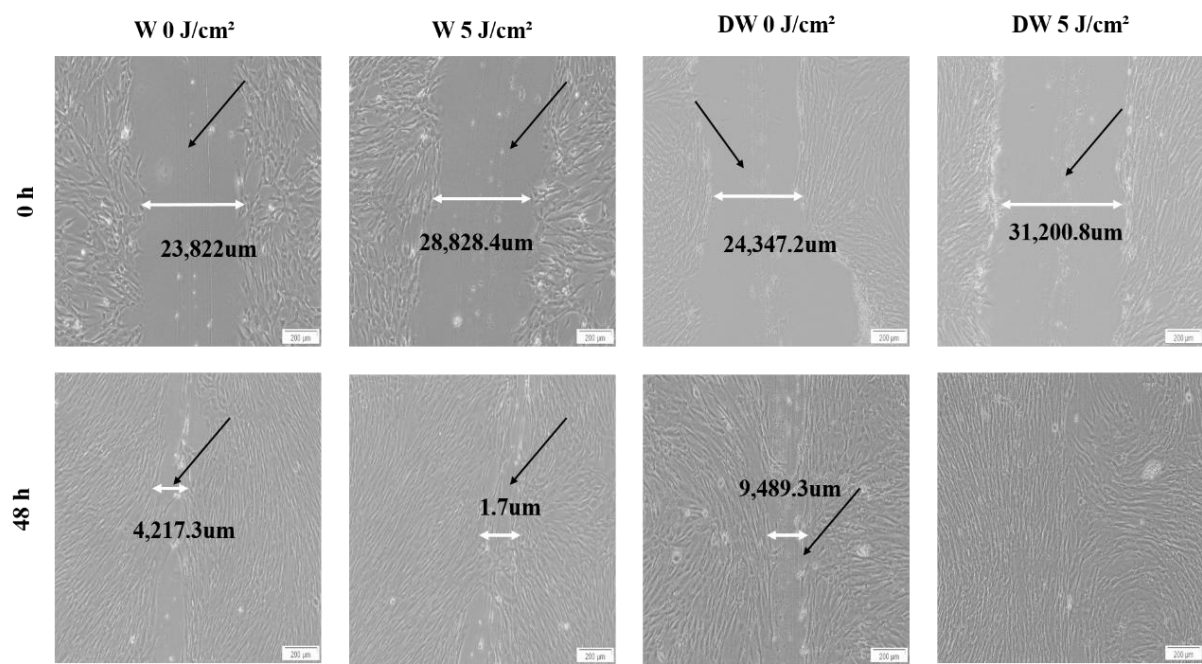


Fig. 2 Cell migration observed at 0 h and 48 h ($n = 3$) in both irradiated (5 J/cm^2) and unirradiated (0 J/cm^2) wounded (W) and diabetic wounded (DW) cells. In the micrographs, the “wound” and the remaining gaps of the wound are shown by a white line or black arrows. Magnification $\times 200$.

Table 2 The percentage migration rate in wounded (W) and diabetic wounded (DW) cells was calculated using the formula $(At_{0h} - At_{time})/At_{0h} \times 100$. An elevated percentage at which the “wound” closed was noted in irradiated (5 J/cm^2) W and DW cells at 48 h ($n = 3$). The p -value (SEM) shows the significant probability as compared to respective unirradiated (0 J/cm^2) controls (SEM).

Model	Unirradiated (0 J/cm ²)	Irradiated (5 J/cm ²)	<i>p</i> -value
W	82.3 ± 1%	99.9 ± 0.1%	<i>p</i> < 0.05
DW	61.0 ± 0.6%	100.0 ± 0.0%	<i>p</i> < 0.01

However, a significant decrease was still evident in irradiated (5 J/cm^2) DW cells ($p < 0.05$) when compared to unirradiated (0 J/cm^2) N cells. A significant decrease in cell viability was noted when unirradiated (0 J/cm^2) DW cell models were compared to unirradiated W cells ($p < 0.01$), as is expected. This decrease was still evident after irradiation ($p < 0.05$).

The MTT assay revealed a significant reduction in viability, with a large effect size (Cohen’s d of 6.496) in irradiated (5 J/cm^2) N cells, and a significant increase in irradiated (5 J/cm^2) W and DW cells with large effect sizes (Cohen’s d of 7.167194 and 4.147085, respectively) (Fig. 3). Using one-way ANOVA, a significant decline in cell viability was observed in unirradiated (0 J/cm^2) W and DW cells when compared to unirradiated (0 J/cm^2) N cells ($p < 0.001$). There was no significant difference observed in cell viability when unirradiated (0 J/cm^2) DW cell models were compared to unirradiated W cells. However, a significant increase in cell viability was

noted in irradiated (5 J/cm^2) DW cells ($p < 0.01$) when compared to irradiated (5 J/cm^2) W cells.

3.3 Apoptosis

Apoptosis normally occurs during embryonic development and is important for maintaining tissue homeostasis. Unwanted compromised cells activate an intracellular death program characterised by the cells morphologic changes including loss of plasma membrane symmetry, condensed cytoplasm and nucleus, and cleavage of deoxyribonucleic acid (DNA). In wound healing apoptosis is critical for the removal of the inflammatory response cells, and later for scar construction [23]. Abnormal and uncontrolled cellular apoptosis may hinder the wound healing process leading to the development of wound chronicity. Here, cellular apoptosis was assessed using the Caspase-Glo® 3/7 assay, and ELISA for the cellular release/excretion of Bcl-2, a cellular protein that controls apoptosis.

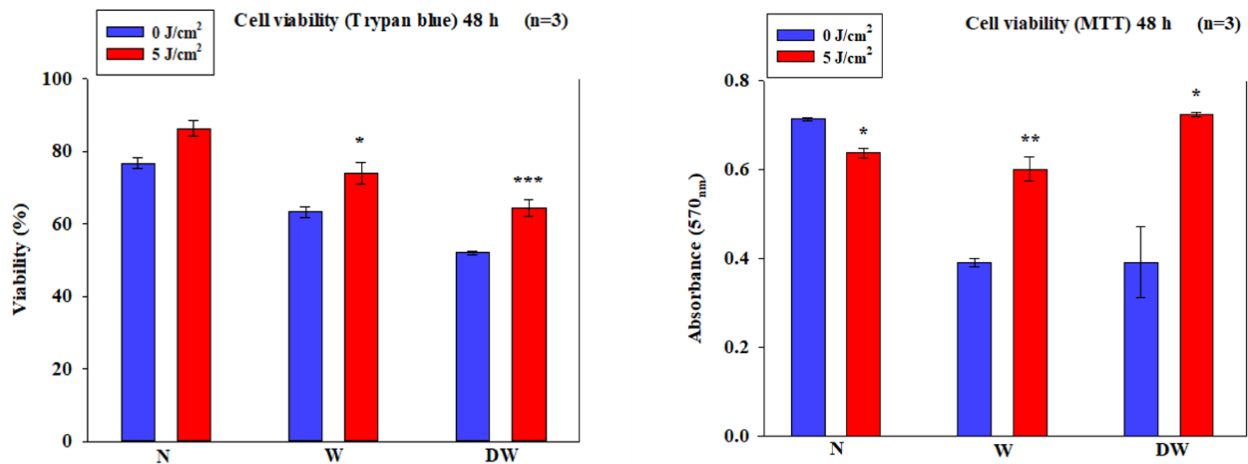


Fig. 3 Cellular viability as measured by the Trypan blue exclusion and MTT assays in unirradiated (0 J/cm²) and irradiated (5 J/cm²) normal (N), wounded (W) and diabetic wounded (DW) cell models 48 h after irradiation. Significant probability is indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ ($\pm$ SEM).

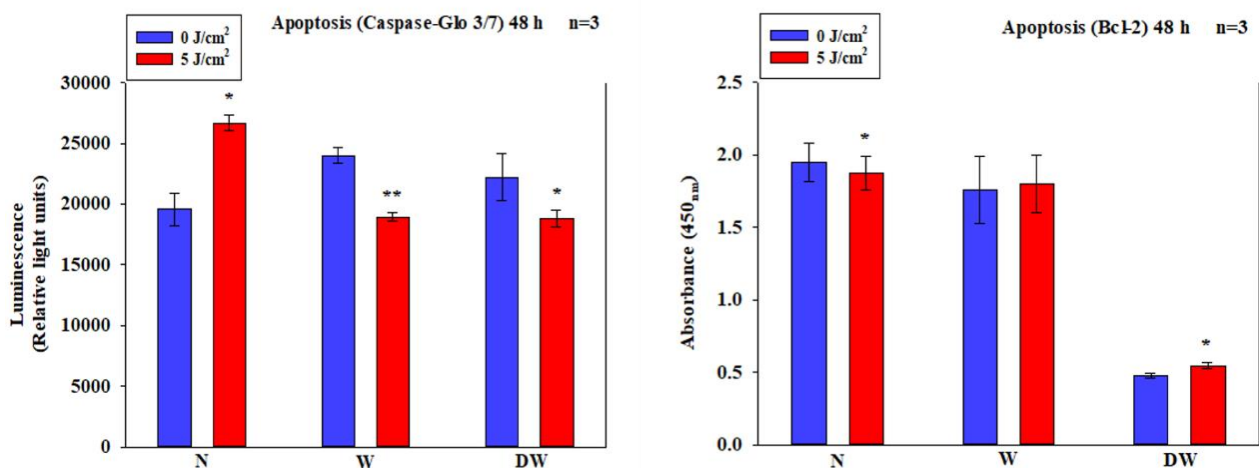


Fig. 4 Cellular apoptosis as measured by the Caspase-Glo® 3/7 assay that measures caspase-3 and -7 activity, and the release/excretion of Bcl-2 (ELISA) in normal (N), wounded (W), and diabetic wounded (DW) cells at 48 h after irradiation at 660 nm with 5 J/cm². Significant probability related to control cells is shown as * $p \leq 0.05$ and ** $p \leq 0.01$ ($\pm$ SEM).

This study further tracked cellular apoptosis using Annexin V/PI assay, a method that is widely used to determine stages of apoptotic cell death. The assay assesses cells that are viable/live, those that are undergoing early apoptosis, and finally those that are undergoing late stage of apoptosis or are dead (necrotic) [24].

3.3.1 Caspase-3 and -7 Activity

Using the Caspase-Glo® 3/7 assay, a significant increase in caspase-3 and -7 activity was observed in irradiated N cells with a large effect size (Cohen's d of 4.755). However, irradiated W and DW cells displayed a significant decrease with large effect sizes (Cohen's d of 6.743 and 1.641, respectively) (Fig. 4). When W and DW cell models were compared to unirradiated (0 J/cm²) N cells using one-way ANOVA, a significant increase in cell caspase-3 and -7 activity was seen in unirradiated (0 J/cm²) W and DW cells ($p < 0.05$). This difference compared to unirradiated (0 J/cm²) N cells was no longer

evident when W and DW cells were irradiated. There was no difference between unirradiated (0 J/cm²) and irradiated (5 J/cm²) W and DW cells, respectively.

3.3.2 Release of Bcl-2

Determining for the release of Bcl-2 into cell culture media using ELISA revealed a significant reduction in Bcl-2 in irradiated (5 J/cm²) N cells with a medium effect size (Cohen's d of 0.587), and a significant increase in irradiated (5 J/cm²) DW cells with a large effect size (Cohen's d of 3.462) (Fig. 4). A significant reduction in the release of Bcl-2 was revealed in both unirradiated (0 J/cm²) and irradiated (5 J/cm²) DW cells ($p < 0.001$) compared to unirradiated (0 J/cm²) N cells. This difference was not observed in unirradiated (0 J/cm²) and irradiated W cells compared to unirradiated (0 J/cm²) N cells. When compared to unirradiated (0 J/cm²) and irradiated (5 J/cm²) W cells, a significant decrease was observed in unirradiated (0 J/cm²) and irradiated (5 J/cm²) DW cells ($p < 0.001$), respectively.

Table 3 Variations in live cells, early apoptosis, late apoptosis, and necrotic cells at 48 h ($n = 3$) using Annexin V/PI–fluorescein isothiocyanate (FITC) and propidium iodide (PI) flow cytometry assay in normal (N) wounded (W) and diabetic wounded (DW). Significant probability is shown as $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ (SEM).

Model	Live cells		Early apoptosis		Late apoptosis		Necrotic cells	
	0 J/cm ²	5 J/cm ²	0 J/cm ²	5 J/cm ²	0 J/cm ²	5 J/cm ²	0 J/cm ²	5 J/cm ²
N	80.0 ± 2.0	82.0 ± 2.0	4.0 ± 0.7	3.0 ± 0.8	13.0 ± 5.0	14.0 ± 1.0	3.0 ± 1.0	1.0 ± 1.0
W	85.0 ± 3.0	85.0 ± 2.0	5.0 ± 0.7	2.0 ± 1.2*	9.0 ± 1.2	17.0 ± 1.2**	2.0 ± 0.5	1.0 ± 0.2
DW	72.0 ± 2.0	83.0 ± 2.0***	9.0 ± 2.0	3.0 ± 1.0**	9.0 ± 0.6	6.0 ± 1.0***	10.0 ± 1.0	6.0 ± 1.0*

The Annexin V/PI assay (Table 3) revealed a significant decrease in the population of early apoptotic cells with a large effect size (Cohen’s d of 1.897), and a significant increase in late apoptotic cells with a large effect size (Cohen’s d of 3.578) in irradiated W cells when compared to their unirradiated control cells. Irradiated DW cells showed a significant increase in live (viable cells) with a large effect size (Cohen’s d of 3.667), and a significant decrease in late apoptotic and necrotic cells with a large effect size (Cohen’s d of 3.667 and 2.530, respectively) when compared to their control cells. When compared to the unirradiated (0 J/cm²) N cell model using one-way ANOVA, a significant increase in cells going through early apoptosis and necrosis was noted in unirradiated (0 J/cm²) DW cells ($p < 0.01$), along with a significant decrease in live cells ($p < 0.01$). There was a significantly increased number of necrotic cells noted in irradiated (5 J/cm²) DW cells ($p < 0.05$) when compared to unstressed, unirradiated (0 J/cm²) N cells. When compared to both unirradiated (0 J/cm²) W cells, unirradiated (0 J/cm²) DW cells showed a significant decrease in live cells ($p < 0.001$). No difference was observed in irradiated (5 J/cm²) DW cells. A significant decrease in late apoptosis was noted in unirradiated (5 J/cm²) DW cells when compared to irradiated (5 J/cm²) W cells ($p < 0.01$). A significant decrease in late apoptosis was noted in irradiated (5 J/cm²) DW cells ($p < 0.001$) when compared to irradiated (5 J/cm²) W cells. No difference was observed in unirradiated (5 J/cm²) DW cells ($p < 0.001$) when compared to unirradiated (5 J/cm²) W cells. There was a significant increase in necrotic cells in both unirradiated (0 J/cm²) and irradiated (5 J/cm²) DW cells when compared to both unirradiated (0 J/cm²) and irradiated (5 J/cm²) W cells ($p < 0.001$), respectively.

4 Discussion

All complications related to DM, including chronic wounds, increase economic burden to the affected persons as well as society [25, 26]. In DM, the normal wound healing process is disrupted, causing tissue damage, and abnormal apoptotic ability [27]. Hyperglycaemia and the systemic build-up of AGEs affect fibroblast cell migration, proliferation, viability, secretion of growth factors, and construction of granulation tissue. Fibroblasts from diabetic patients suffer mitochondrial impairments and become

susceptible to apoptosis [28, 29]. PBM has shown to have therapeutic effects on diabetic wounds, and when used as an adjunctive therapy along with standard treatment methods, it improves diabetic wound healing [30, 31]. For instance, when Layegh et al. [32], used PBM at a wavelength of 660 nm and a fluence of 3 J/cm², they noted a significant decrease in the diabetic wound area and an increase in the formation of granulation tissue. Nevertheless, the mechanism of PBM in diabetic wound healing is not completely clarified. This study used PBM at 660 nm and a fluence of 5 J/cm² to understand its molecular and cellular effect on diabetic wounded fibroblast cells 48 h after irradiation.

Under a light microscope, fibroblast cells appear spindle shaped or radiating star like patterned cells with a centrally round or oval nucleus [33]. According to Loots et al. [34], fibroblast cells from diabetic foot ulcers appear to be large and broadly spread under light microscopy. Chellini et al. [35], observed that near infrared (NIR) and violet-blue light irradiated fibroblast cells presented with a polygonal outline and a higher surface area when compared to red light irradiated fibroblast cells which seemed extended or stretched and spindle-shaped. The present study noticed no cellular morphological difference in both irradiated and non-irradiated normal and diabetic cell models following irradiation. Gradually, cells began spreading out and changed their direction of growth, and started moving into the central scratch in an attempt to ‘close the wound’.

Fibroblast cells are a key in the production of ECM, and remodelling and tightening of the healing wound [36]. Mostly, wound healing is delayed or may not get restored at all when the granulation tissue construction process is dysfunctional [37]. Fibroblast cell migration towards the wound milieu is critical during the proliferative stage of wound healing. In DM, cellular migration has been shown to be delayed [38]. The present study demonstrates that PBM at 660 nm and a fluence of 5 J/cm² accelerates the wound repairing processes via the stimulation of fibroblast migration and viability. The study observed a significant increase with a large effect size in cellular viability, migration towards the centre of the “wound”, and wound closure in irradiated wounded and diabetic wounded cell models. Xuan et al. [39], suggested that the hyperglycaemic mediated suspension of cell migration is related to the inhibition of basic fibroblast growth factor (bFGF) signalling, specifically because of c-Jun N-terminal kinases (JNKs) suppression.

In another study, Lerman et al. [40], observed that diabetic fibroblasts show impairments in cellular migration, growth factor production, and their reaction to hypoxia *in vitro*. This study demonstrated decreased cell migration and viability in unirradiated DW cells when compared to unirradiated W cells, indicating the effect of high glucose concentration in the culture media. However, after irradiation this effect was no longer evident, suggesting the positive effect of PBM on diabetic cells and the effect of high glucose concentration in the culture media.

Besides reducing cell migration, viability, proliferation, and synthesis of collagen, *in vitro* experiments have shown that hyperglycaemia increases apoptosis in different cell types [41]. In typical wound healing, apoptosis is necessary for eradicating inflammatory cells and scar construction without inflammation and tissue damage [30], and involves different cellular morphological features and signalling pathways with the instigation of caspases, and subsequent reduction in viability [42]. PBM at a wavelength ranging between 600 and 1200 nm has been reported to reduce apoptosis in different tissues, and when Davies et al. [43], investigated the influence of 660 nm PBM on cell apoptosis, they observed a significantly high fraction of apoptotic cells in the control group as compared to irradiated group. In addition, Rajendran et al. [44], noted that PBM at a wavelength of 660 nm reversed high oxidative stress in diabetic and diabetic wounded fibroblast cells as represented by decreased FOXO1 levels, and suggested that PBM advanced diabetic wound healing by reducing oxidative stress via the inhibition of FOXO1 signalling. Tam et al. [45], suggested that PBM at a lower energy reduces apoptosis but promotes it at a higher energy. Nonetheless, the existing understanding regarding the effect of PBM on cellular apoptosis and diabetic wound healing is elusive. To determine the protective impact of PBM at 660 nm and 5 J/cm² against apoptosis in diabetic wound healing, this study used Caspase-Glo® 3/7 assay, ELISA and Annexin V/PI assay to compare cellular caspase activity, secretion of Bcl-2, the percentage of live cells, and those that were undergoing early and late apoptosis, as well as necrotic cells in both unirradiated and irradiated cell models. This study noted a significant decrease with a large effect size in the activity of pro-apoptotic caspase-3 and -7, and a significant increase with a large effect size in anti-apoptotic Bcl-2 protein in DW cells exposed to PBM at 660 nm. This study noted an increase in the percentage of cells undergoing early apoptosis in unirradiated DW cells as compared to unirradiated W cells, signifying the effect of high sugar concentration in the culture media. As compared to unirradiated N cells, there was a considerable rise in early

apoptosis and necrotic cells, as well as a decrease in viable cells and late apoptosis in unirradiated W and DW cells. However, when these cells were irradiated, they responded positively with a large effect size, as compared to their unirradiated control cells, suggesting a positive therapeutic effect of PBM. In another study, Cheng et al. [46], suggested that PBM improves cell proliferation and reduces tumour necrosis factor alpha (TNF- α)/cycloheximide-facilitated cell death. Maldaner et al. [47], also suggested that PBM augmented the proliferative and protective effect of hydrogen peroxide (H₂O₂) induced senescence in fibroblast cells. These observations indicate that the therapeutic effects observed were due to PBM, and this study has further demonstrated that PBM used at a wavelength of 660 nm and a fluence of 5 J/cm² influence cell viability, migration, and wound healing specifically through modulation of the cellular pro- and anti-apoptotic proteins in diabetic wounded cells *in vitro*.

5 Conclusion

This study has demonstrated that reduced wound healing in diabetic wounded cells *in vitro* is caused by reduced cell viability and migration, and increased cell apoptosis. However, PBM effectively regulated these defects, and restored the normal functioning of DW cells *in vitro*, indicating that PBM in the visible red range speed up diabetic wound healing. Although the present study demonstrated these molecular effects of PBM in hastening wound healing, there is a need for further studies to gain more understanding on its effect at a molecular, cellular and tissue level. This study has exhibited that PBM could indeed influence the rebuilding and restoration of diabetic wounds, and within this context, it could be used as an adjunct treatment method to stimulate wound healing processes *in vivo*.

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Disclosures

The authors declare no conflict of interest.

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