A comparison between photobiomodulation at 830 nm and 660 nm on differentiation in diabetic human skin fibroblast cells

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Abstract. Different studies have proposed the efficacy of photobiomodulation (PBM) at different wavelengths (830 and 660 nm) to stimulate wound repair in diabetic cells. The TGF-β1/Smad cascade has proven to be an effective signalling pathway in differentiating fibroblasts into myofibroblasts. This study aims to compare the effects of both wavelengths on cellular viability and expression of fibroblast differentiation markers in WS1 fibroblast cells. The cells were modelled into groups; normal (N), normal wounded (NW) and diabetic wounded (DW). At 830 nm and 660 nm, cells were irradiated with 5 J/cm^2, while control cells were without irradiation (0 J/cm^2). At 24 and 48 h post-irradiation cell viability was investigated using the Trypan blue exclusion assay, while transforming growth factor-beta (TGF-β1) and p-Smad2/3 was ascertained using ELISA. Immunofluorescence was used to observe the presence of alpha smooth muscle actin (α-SMA). There was a significant increase in cell viability in the irradiated models using both wavelengths. A wavelength of 830 nm elicited a slight increase in TGF-β1 compared to 660 nm in diabetic wounded cells, while both wavelengths had no effect on p-Smad2/3. Both wavelengths were successful in initiating the differentiation of fibroblasts into myofibroblasts in diabetic wounded cells with no difference between wavelengths.

1. Introduction

Diabetes mellitus (DM) remains a complex metabolic disorder, with symptoms that progress to diverse micro and macro- complications. Presently, over 463 million cases of diabetes have been recorded globally [1]. This implies that 1 in 11 people worldwide currently have diabetes. The number of diabetes cases is estimated to increase to 700 million by 2045 (51% increase) [1]. In South Africa, the prevalence of diabetes surged from 5.5% to 9% within 2000 and 2010, making the country the second highest with respect to mean health care expenditure in the African region [2].

A rise in blood glucose levels occurs in diabetes due to insulin resistance which then leads to energy starvation and inadequate utilisation of sugar in peripheral organs. Hyperglycaemia has been implicated in the slow healing of wounds seen in diabetic patients with chronic foot ulcers [3, 4]. As a result, the quality of life of diabetic patients is often impacted negatively, not only because of their limited mobility and increased risk of death from limb amputations, but also due to the treatment costs that would have been incurred. Globally, reports indicate that a lower limb amputation occurs in

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diabetic patients every 30 seconds [5]. New and potent modalities are therefore required to accelerate the healing process of diabetic wounds to reduce the risk of amputation and increase the quality of life of patients.

There is an urgent need to identify effective ways to accelerate the wound healing process, thereby reducing the global burden of diabetic wounds. Light (including lasers) can be used to speed up healing in a process called photobiomodulation (PBM), but the activation of specific and important cellular pathways in response to PBM must be identified to make the treatment more lucrative [6]. PBM is a non-invasive and painless therapy and can be used singly or jointly with other treatment strategies to speed up the healing of diabetic wounds. The positive influence of PBM on wound healing at *in vitro* level has been reported in earlier studies using stem cells, keratinocytes, fibroblasts, mast cells, osteoblasts, and others [7, 8]. *In vitro* PBM (adopting wavelengths of 630 to 830 nm with a fluence from 2 to 5 J/cm^2) on diabetic cells has elicited positive responses to induce effective wound healing [9, 10, 11].

The inflammatory phase of wound healing becomes defective in diabetes, leading to downregulation of growth factors, including transforming growth factor beta-1 (TGF-β1) [6] and alteration of differentiation of cells (such as fibroblasts) necessary for wound closure [7]. Usually, fibroblast cells cover the wound area differentiating into extra domain-A fibronectin (EDA-FN) expressing proto-myofibroblasts, which finally differentiate into alpha smooth muscle actin (α-SMA) expressing myofibroblasts which aids in wound contraction through the Smad pathway. The Smad3 cascade remains a novel and unexplored area in response to PBM.

This study aims to compare the influence of PBM at different wavelengths (830 nm and 660 nm) on cellular viability and presence of fibroblast differentiation markers in normal and diabetic WS1 fibroblast cells.

2. Materials and Methods

A commercially purchased human skin fibroblast cell line (WS1, ATCC® CRL-1502™) was cultured aseptically in line with the supplier's protocol. Three models, namely normal (N), normal wounded (NW) and diabetic wounded (DW) were used. Diabetic cells were attained by continually growing the cells in complete MEM (minimal essential media) containing D-glucose (17 mMol/L) to achieve and maintain hyperglycaemia [12]. Cells ($6x10^5$) were transferred into 3.4 cm diameter culture plates. To achieve a wound, a scratch was created with a sterile 1 mL pipette on a confluent monolayer of cells [13]. Irradiation occurred at 830 nm and 660 nm using a diode laser at a fluence of 5 J/cm^2 while non-irradiated cells (0 J/cm^2) served as controls. Laser parameters used are recorded in table 1.

Post-irradiation, incubation of cells was done for 24 and 48 h. The viability of the cells was ascertained using the Trypan blue exclusion assay. The human TGF-β1 ELISA kit (Whitehead Scientific, R&D Systems, DY240) was utilized to quantify the amount of released TGF-β1 in the culture medium while the phosphorylated-Smad2/3 (Ser423/425) ELISA kit (Thermo Fisher Scientific, Invitrogen, 85-86192) was used to determine the phosphorylation (and hence activation) of Smad2/3 in the cells. Both experiments were done using the ELISA kits' protocols, and colorimetric reactions were quantified spectrophotometrically at 450 nm (Victor3 multiplate reader; Perkin-Elmer).

Immunofluorescence was performed by fixing cells at room temperature on a coverslip with 4% paraformaldehyde for 15 min followed by permeabilization with 0.5% triton X-100 in phosphate buffered saline. The first labelling of cells was done with anti-human alpha-Smooth Muscle Actin (Whitehead Scientific, R&D Systems, MAB1420) a primary antibody, followed by a washing step. Then the second labelling was done using a fluorescently tagged secondary antibody (Anti-mouse IgG NL557 conjugated secondary antibody; Whitehead Scientific, Novus Biological, NL007). Post-washing, counter staining of the nuclei was done with 1 µg/mL 4',6-diamidino-2-phenyndole (DAPI) followed by mounting onto a slide and viewing on the Carl Zeiss Axio Z1 Observer using Zen imaging software.

Samples were analysed three times (n=3) and statistical analysis was done using SPSS version 27. The student t-test and One-way ANOVA was used to determine statistical differences and results were considered significant when $p < 0.05$.

Table 1. Laser parameters.

Variables		
Wavelength (nm)	830	660
Light source	Diode laser	Diode laser
Wave emission	Continuous wave	Continuous wave
Spot Size (cm^2)	9.1	9.1
Power Output (mW)	105	100
Power density (mW/cm^2)	11.54	11
Irradiation time	7 min 13 s	7 min 34 s
Energy density (J/cm^2)	5	5
Energy (J)	45.5	45.4

3. Results and Discussion

The high costs associated with the treatment of diabetic wounds causes a strain on the global expenditure on diabetes. In diabetes, slow healing of wounds occurs due to a decrease in the production of collagen; the main content in the extracellular matrix (ECM) [14, 15]. The migration of fibroblast cells to the wound area during the normal wound healing process induces cytokine and growth factor secretion in the ECM [15]. Research has shown that PBM is effective in increasing the rate of diabetic wound healing through stimulation of cellular processes with little side effects. This could be through the increased expression of α-SMA. Application of red (660 nm) and near-infrared (830 nm) laser in PBM has emerged as a promising technique for speeding up the wound healing process, minimising pain, and improving skin function due to direct wound penetration and modulation of biochemical pathways [16].

Results show an increase in cell viability in the tested models (figure 1). This shows the success of PBM using both wavelengths in promoting cell viability, as has been shown in other studies [17, 18]. In N cells, irradiation at 830 nm elicited an increase in cellular viability compared to 660 nm after 24 h ($p < 0.001$) and 48 h ($p < 0.05$). There was no substantial variance in viability of the NW cells upon comparison of both wavelengths at 24 and 48 h. However, the DW cells irradiated at 830 nm exhibited a considerable increase in viability ($p < 0.01$) in comparison to the same cells irradiated at 660 nm at 48 h.

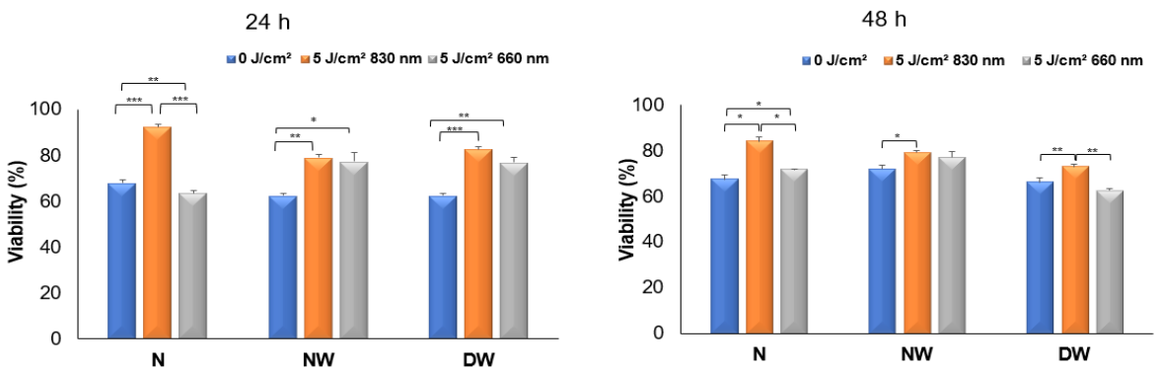

Figure 1. Cellular viability (%), assessed via the Trypan blue exclusion assay. Viability was determined in non-irradiated (0 J/cm^2) and irradiated (5 J/cm^2 at 830 nm and 660 nm) normal (N), normal wounded (NW), and diabetic wounded (DW) cells, and analysed 24 and 48 h post-irradiation. Statistical significance is presented as *P<0.05, **P<0.01 and ***P<0.001 (±SEM).

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In comparison to irradiation at 830 nm, irradiation at 660 nm resulted in a considerable ($p < 0.05$) increase in p-Smad 2/3 levels at 24 and 48 h in all the tested cell models (figure 2). This could suggest that irradiation at 660 nm might have initiated the activation of the Smad signalling pathway which was not noticed using the 830 nm. Interestingly, irradiation at 830 nm showed an increase in TGF-β1 compared to 660 nm (table 2). This might be due to differentiation occurring before 24 h in the 660 nm irradiated cells, thereby causing the cells to use up more TGF-β in a paracrine fashion. TGF-β has been reported to increase the wound healing process by stimulating the production of collagen by fibroblast cells [19].

The immunofluorescence results using 660 nm showed an increase in the presence of α-SMA at 24 and 48 h post-irradiation compared to using 830 nm (figure 3). In skin contraction, during wound healing, myofibroblasts yield α-SMA to signify full fibroblast differentiation [20, 21]. Studies have shown that PBM using both wavelengths can trigger the release of α-SMA during wound healing [22, 23].

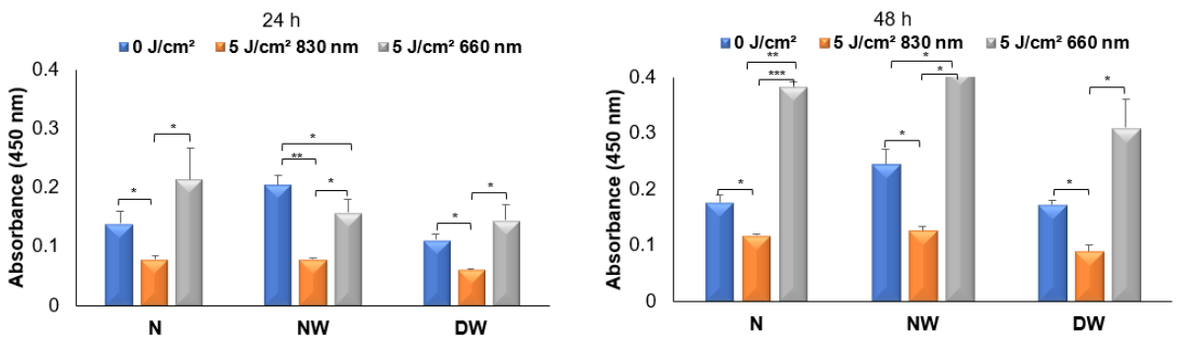

Figure 2. The presence of p-Smad2/3 was monitored using ELISA at 24 and 48 h in normal (N), normal wounded (NW) and diabetic wounded (DW) WSI fibroblast cells irradiated with an 830 nm and 660 nm laser at 5 J/cm^2 while the control group was not irradiated (0 J/cm^2). Statistical significance is presented as *P<0.05, **P<0.01 and ***P<0.001 (±SEM).

Table 2. The presence of TGF-β1 was measured using ELISA at 24 and 48 h in normal (N), normal wounded (NW) and diabetic wounded (DW) WS1 fibroblast cells irradiated with an 830 nm and 660 nm laser at 5 J/cm^2 while control group was not irradiated cells (0 J/cm^2).

	24 h			**48 h**		
	Control (0 J/cm²)	5 J/cm² 830 nm	5 J/cm² 660 nm	Control (0 J/cm²)	5 J/cm² 830 nm	5 J/cm² 660 nm
N	0.81 ± 0.03	1.49 ± 0.07**††	0.02 ± 0.0006***	0.85 ± 0.02	1.81 ± 0.01***†††	0.03 ± 0.007***
NW	0.74 ± 0.04	1.48 ± 0.07**††	0.02 ± 0.002**	0.87 ± 0.01	1.63 ± 0.02**†††	0.02 ± 0.0005***
DW	0.62 ± 0.01	1.37 ± 0.03**†††	0.021 0.0002***	0.76 ± 0.005	1.73 ± 0.02***†††	0.02 ± 0.001***

Statistical significance as compared to the control is shown as **P<0.01 and ***P<0.001, † signifies statistical difference between irradiation at 830 nm (5 J/cm²) and irradiation at 660 nm (5 J/cm²) (±SEM).

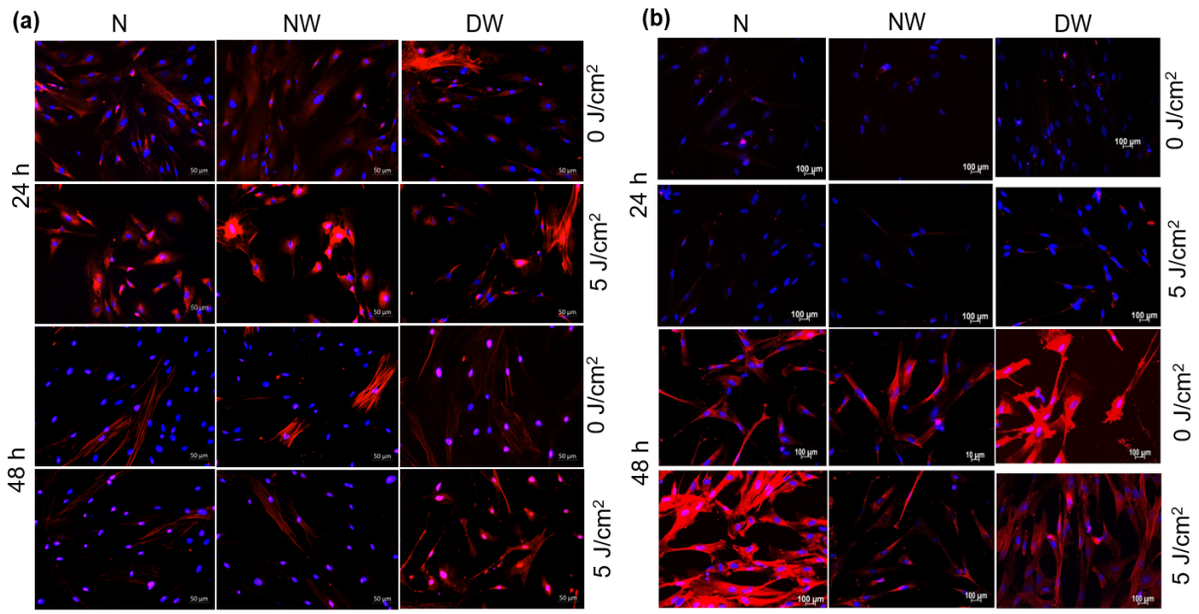

Figure 3. (a) Irradiation at 830 nm and **(b)** 660 nm wavelengths in normal (N), normal wounded (NW) and diabetic wounded (DW) cells irradiated at a fluence of 5 J/cm^2 and control cells (0 J/cm^2). Cells were incubated for 24 and 48 h and stained for α-SMA (red). Counterstaining of nuclei was done with DAPI (blue), magnification (x200).

4. Conclusion

The study was aimed at comparing the influence of PBM at 830 nm and 660 nm on differentiation of N, NW and DW fibroblasts into myofibroblasts 24 and 48 h after irradiation. The results discussed showed that PBM at both wavelengths influenced the cells in comparison with the non-irradiated cells. There was an increase in the presence of p-Smad and α-SMA when using a wavelength of 660 nm in comparison with 830 nm. This difference may be due to the fact that the process of differentiation occurred faster at 660 nm (than at 830 nm) and could be the reason for the low presence of TGF-β1 (at 660 nm) which could have already been used up by the cells during differentiation. Both wavelengths are equally successful in *in vitro* wound healing assays. More experiments (probably *in vivo* experiments) need to be done before we can justify the bases for a better wavelength.

Acknowledgments

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