The effect of photobiomodulation at 660 nm on the differentiation of diabetic wounded WS1 human fibroblasts into myofibroblasts

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Abstract. Diabetes is associated with complicated wound healing and rapid wound progression which may be due to cells that fail to proliferate and differentiate, thus leading to ulcers and possible limb amputation. The administration of photobiomodulation (PBM) has been associated with increased cellular proliferation, a decrease in wound repair duration and an increase in wound flexibility. Most studies performed on PBM and myofibroblasts dwell mostly on fibrosis, and a minority of studies have investigated fibroblast differentiation for use in diabetic wound healing. This study aimed to determine the effect of PBM at 660 nm with 5 J/cm^2 on the differentiation of diabetic wounded human fibroblasts 48 h post-irradiation. This was achieved by measuring the expression of the fibroblast surface marker Thy-1 (CD90), proto-myofibroblast marker EDA fibronectin (EDA-FN), and the myofibroblast marker alpha smooth muscle actin (α-SMA) by immunofluorescence and flow cytometry. Post-PBM, there was a significant decrease in Thy-1, and an increase in EDA-FN and α-SMA, indicating the transformation of fibroblasts during wound healing. Photobiomodulation at 660 nm with 5 J/cm^2 stimulates cellular differentiation of diabetic wounded fibroblast cells into myofibroblasts, which contributes to the increased rate of wound healing.

1. Introduction

Diabetes Mellitus (DM) is a common metabolic disorder resulting from the lack of or resistance to insulin by cells [1]. As a result, blood glucose levels are high, which leads to a number of complications such as inflammation, nephropathy, retinopathy, neuropathy and cardiovascular disease [2], as well as delayed wound healing. Globally, DM is a growing problem and poses an economic burden to all countries [3]. In Africa alone, the number of cases of DM is expected to increase by 162.5% by 2045. Individuals are at risk of limb amputations in their productive years due to impaired wound healing, resulting in an increased economic burden, as well as reduced quality of life. It is estimated that a lower limb or part of the lower limb is lost in every 30 seconds worldwide [2].

The normal wound healing process is characterized by three overlapping phases namely: inflammation, proliferation and remodeling. In diabetic or chronic wounds, the wound healing process remains trapped in the inflammation phase and cells do not differentiate to aid in wound closure. Fibroblasts are one of the cells that play a major role in wound healing. They infiltrate the wound site and differentiate into myofibroblasts which are liable for wound contraction. They also produce most of the extracellular matrix (ECM), collagens, matrix metalloproteinase (MMPs), tissue inhibitors of the matrix metalloproteinase (TIMPs) and glycosaminoglycans (GAGs) [4]. Fibroblasts express the cell

surface marker CD90 or Thy-1. During wound healing they differentiate into extra domain-A-fibronectin (EDA-FN) expressing proto-myofibroblasts, which finally differentiate into alpha smooth muscle actin (α-SMA) expressing myofibroblasts via the Smad pathway [5]. The presence of EDA-FN aids in the complete differentiation into myofibroblasts [6]. It is believed that in diabetic wounds this differentiation does not occur as the wound healing process remains stagnant in the inflammatory phase [7].

Photobiomodulation (PBM) is a non-invasive, non-thermal therapy which involves the application of red or near-infrared (NIR) light to stimulate, regenerate, protect and heal injured or degenerating tissues [8]. Its exact mechanism is not fully understood. However, it is known that PBM activates photo-reactive proteins such as cytochrome C oxidase in the mitochondrion's respiratory sequence, thus increasing the presence of adenosine triphosphate (ATP) in the cells [9]. This increase has been found to normalize cellular functions, decrease pain and activate the healing process. Most studies performed on PBM and myofibroblasts dwell mostly on fibrosis, and a minority of studies have investigated fibroblast differentiation for use in diabetic wound healing. The aim of this study was to investigate the effect of PBM on the differentiation of fibroblasts into myofibroblasts in diabetic induced wound healing *in vitro*.

2. Materials and methods

Human skin fibroblast cells (WS1, ATCC® CRL-1502™) were grown according to standard techniques. Cells were continuously grown in minimal essential media (MEM) containing 10% foetal bovine serum (FBS), 0.1 mM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 1% Amphoterisin B, 1% penicillin-streptomycin, 2 mM L-glutamine, and to diabetic cultures 17 mMol/L D-glucose was added to achieve a diabetic model [10, 11]. Cells ($6x10^5$ for immunofluorescence, and $1 x10^6$ for flow cytometry) were seeded into 3.4 cm^2 culture plates. A wound model was created via the scratch assay, in which a confluent monolayer of cells was scrapped with a sterile 1 mL pipette. Cells were irradiated with a 660 nm diode laser at a fluence of 5 J/cm^2 in the dark from above with the culture dish lid off. Laser parameters are shown in Table 1. This was followed by incubation for 48 h. Non-irradiated cells were used as controls (0 J/cm^2). Immunofluorescence and flow cytometry techniques were performed.

For flow cytometry, harvested cells were pelleted and fixed with 0.5 mL cold Flow Cytometry Fixation Buffer (FC004, R&D Systems, Whitehead Scientific, South Africa) and incubated at room temperature for 10 min. Following two washes with phosphate buffered saline (PBS), pelleted cells were re-suspended in 150 µL Flow Cytometry Permeabilization /Wash Buffer I (FC005, R&D Systems, Whitehead Scientific, South Africa) and incubated for 30 min at room temperature in 10 µL Phycoerythrin (PE)-conjugated antibody (mouse, anti-Human CD90\Thy1, FAB2067P; mouse, anti-Human α-smooth muscle actin, IC1420p; R&D Systems, Whitehead Scientific, South Africa). Cells were washed twice with Flow Cytometry Permeabilization /Wash Buffer I and re-suspended in 300 µL PBS for flow cytometric analysis on the BD Accuri C6.

For immunofluorescence, cells were fixed on a coverslip with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% triton X-100 in PBS, and blocked with 1% bovine serum albumin (BSA) in PBS to prevent any non-specific binding. Cells were first labelled with primary antibody (sheep, anti- human CD90/Thy1, AF2067; mouse, anti-human alpha-Smooth Muscle Actin, MAB1420; R&D Systems, Whitehead Scientific, South Africa; and rabbit, anti-human EDA, HPA037972; Sigma-Aldrich, South Africa). After washing, cells were labelled with a fluorescently conjugated secondary antibody (557-conjugated Anti-Sheep IgG Secondary Antibody NL010; or 557-conjugated Anti-Mouse IgG Secondary Antibody, NL007; R&D Systems, Whitehead Scientific, South Africa; or FITC-conjugated Anti-Rabbit IgG antibody, D2706, Santa Cruz, Anatech Instruments (Pty) Ltd, South Africa; respectively). After washing, nuclei were counter stained with 1 µg/mL 4:6-diamidino-2phenyndole (DAPI). The coverslip was mounted onto a slide and viewed on the Carl Zeiss Axio Z1 Observer using AxioVision software.

Statistical analysis was achieved on three repeats of each sample. The t-test was used to determine statistical differences using Sigma Plot version 13.0 (Systat Software Inc., San Jose, California) and was considered significant when $P< 0.05$.

Table 1. Laser parameters.

Variables	Diode laser
Wavelength (nm)	660
Light source	Diode laser
Wave emission	Continuous wave
Spot Size (cm^2)	9.1
Power output (mW)	100
Power density (mW/cm^2)	11
Irradiation time	7 min 6 s
Energy density (J/cm^2)	5

3. Results and discussion

Post-irradiation, Thy-1 immunofluorescence results revealed a decrease in signal for the irradiated diabetic wounded model (DW5J) as compared to the non-irradiated diabetic wounded model (DW0J) (Figure 1). The flow cytometry results showed and confirmed this significant decrease ($P<0.001$) in Thy-1 in DW5J cells (1.2% of cells stained positive for Thy-1) as compared to their control DW0J (3.2% of cells stained positive for Thy-1) (Figure 2). There was an increase in the proto-myofibroblast marker EDA-FN, as well as the myofibroblast marker α-SMA, in the DW5J model compared to the control DW0J 48 h post-irradiation (Figure 1). A significant increase in α-SMA was confirmed in the DW5J model by flow cytometry ($P<0.01$), with 14,2% of the population staining positive for α-SMA as compared to only 4.8% in the DW0J model (Figure 2).

During normal wound healing, fibroblasts migrate into the wound site and secrete cytokines, growth factors and the ECM [5, 12]. Once activated they differentiate into myofibroblasts which also participate in ECM secretion and wound size reduction through contraction [5, 13]. In DM there is little production and quick removal of collagen, which is the main content of the ECM. This leads to debilitated wound healing and cells remaining senescent in the inflammation phase. Maione et al observed that in diabetic wound healing, fibronectin (FN) content is increased and the ECM deposited is thinner in comparison to normal wound healing, they then concluded that the increased FN hindered the secretion of a more mature ECM which is excreted by myofibroblasts [14]. This suggests that in diabetic wound healing differentiation of fibroblasts does not occur, and if it does occur it remains in the proto-myofibroblast phenotype, hence the increased FN. Modern day treatments of diabetic and chronic wounds are associated with failure and relapse which increases the expense of wound care [7]. PBM has been proven to stimulate cellular processes without any harmful effects. It works by stimulating cytochrome C oxidase leading to increased ATP production, cellular regeneration, and enhanced survival and proliferation. Increased ATP is evidenced by the activation of cellular pathways associated with wound healing, cell differentiation and proliferation [8, 15]. A study done on dermal wounded and immunosuppressed rats irradiated at 810 nm showed a reduction in the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α), and an increase in the expression of fibroblast growth factor receptor (FGFR-1) and FN, which enhances the protein expression of α-SMA. This showed that PBM results in an increase in cellular motility and contractility which is linked to an increased expression of α-SMA [16]. In this study, PBM at 660 nm with 5 J/cm^2 has proved to stimulate the transition of fibroblasts into myofibroblasts by decreasing Thy-1, and increasing EDA-FN and α-SMA in irradiated diabetic wounded fibroblasts, which is vital for proper and successful wound healing.

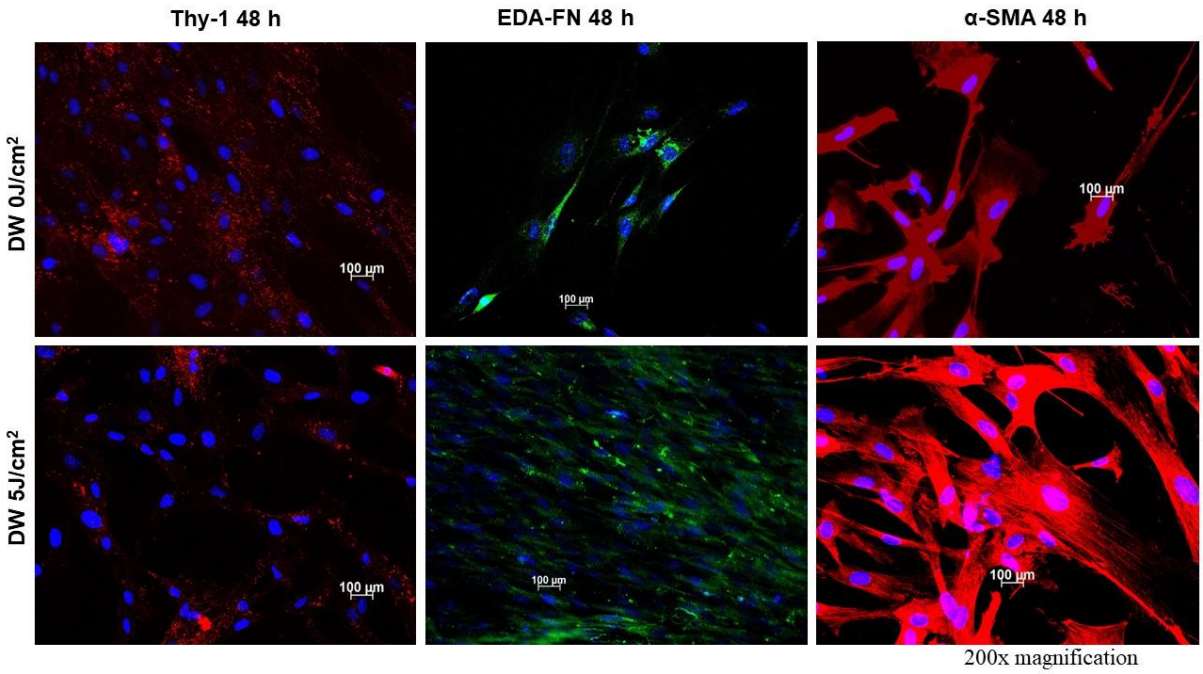

Figure 1. Diabetic Wounded (DW) fibroblasts irradiated with 660 nm at a fluence of 5 J/cm^2 (DW 5Jcm^2) and control cells (non-irradiated 0J/cm^2; DW 0J/cm^2). Cells were incubated for 48 h and stained for the Thy-1 marker with NL557-conjugated anti-sheep IgG antibody (red); EDA-FN was stained with FITC-conjugated Anti-Rabbit IgG antibody (green); and α-SMA was stained with NL557-conjugated anti-Mouse antibody (red). Nuclei were counterstained with DAPI (blue).

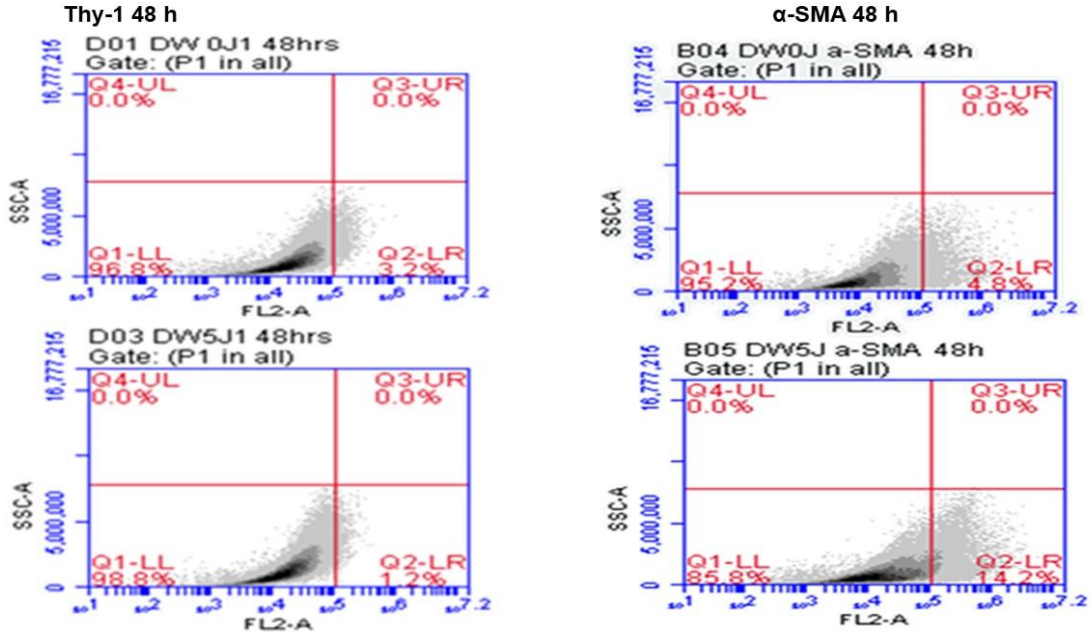

Figure 2. PE stained (mouse, anti-Human CD90\Thy1; mouse; and anti-Human α-smooth muscle actin) WS1 diabetic induced fibroblast cells 48 h post irradiation with 660 nm at a fluence of 5 J/cm^2 (DW5J) and non-irradiated control cells (DW0J). Q1 indicates cells that are negative for the Thy-1 or α-SMA marker, while Q2 indicates cells that are positive for Thy-1 or α-SMA, respectively.

4. Conclusion

The aim of this study was to determine the effect of PBM at 660 nm with 5 J/cm^2 on the differentiation of diabetic wounded fibroblasts into myofibroblasts 48 h post-irradiation. The results obtained indicated that PBM had a positive effect on irradiated diabetic wounded fibroblast cells (DW5J), and therefore resulted in a significantly increased rate of differentiation of fibroblasts into myofibroblasts in comparison with the non-irradiated model (DW0J). Hence, the evident decrease in Thy-1, and increase in EDA-FN and α-SMA expression at 48 h in irradiated cells (DW5J). This transition is important since myofibroblasts play a major role in wound contraction and ECM deposition to achieve successful wound healing. This contributes to the knowledge that PBM has therapeutic properties which enable it to stimulate wound healing in diabetic wounded cells *in vitro*, thus increasing the chances of its application and acceptance as one of the therapeutic modalities in chronic and diabetic wound care.

Acknowledgments

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