PBM at 660 nm reduces stress-induced apoptosis in diabetic wounded fibroblast cells *in vitro*

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Abstract. Uncontrolled diabetes mellitus (DM) increases reactive oxygen species (ROS) and oxidative stress. Oxidative stress provokes apoptosis, a programmed cell death that typically sustains the developmental mechanism for normal body homeostasis. Oxidative damage affects the expression of pro-apoptotic proteins and anti-apoptotic proteins including caspases and B cell lymphoma 2 (Bcl 2). Uncontrolled apoptosis is one of the major causes for the development of chronic diabetic wounds. Photobiomodulation (PBM) involves exposing wounds to light from lasers or light emitting diodes (LEDs) to induce healing. However, its protective mechanisms and the ideal protocol on cellular apoptosis remain unclear. In this investigation, WS1 skin fibroblast cells were split into diabetic (D) and diabetic wounded (DW) cell models and were subjected to a continuous wave diode laser at a wavelength of 660 nm and a fluence of 5 J/cm². Non-treated cells (0 J/cm²) were used as controls. Irradiated cells were incubated for 48 h and evaluated for viability and apoptosis. PBM at 660 nm significantly increased cellular viability and reduced apoptosis in both irradiated D and DW cells. This study suggests that PBM at 660 nm and 5 J/cm^2 increases cell viability and reduces apoptosis.

1. Introduction

Reactive oxygen species (ROS) including hydroxyl radicals ($OH^{\cdot}$), superoxide anion (O_2^{-}), and hydrogen peroxide (H_2O_2) are derivatives of aerobic metabolism, and have characteristic chemical properties that cause reactivity to cells and tissue. Research has related ROS to the development of oxidative stress, which stimulates pathological conditions by affecting proteins, lipids, and cellular DNA. Nevertheless, controlled ROS is critical for cellular signalling and the alteration of biological and physiological activities including growth and development [1]. In addition, regular ROS concentrations are key regulators of all the phases in the progression of wound healing, the fight against microbes, and activates cell survival signalling [2]. To ensure survival in the presence of increased ROS, the human body generates antioxidants, which prevent and slow ROS mediated injury. Two forms of antioxidants including enzymatic components, i.e. catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX), and non-enzymatic antioxidants, i.e. ascorbic acid (AA), glutathione (GSH), carotenoids, and flavonoids work together to scavenge ROS [3]. Oxidative stress comes about due to disproportional production of ROS and antioxidants, and over time, oxidative stress can cause diseases including diabetes mellitus (DM), hardening of the blood vessels

(atherosclerosis), inflammation, high blood pressure (hypertension), heart disease, cancer, and neurodegenerative diseases [4].

DM (a group of metabolic diseases characterised by high blood sugar, also known as hyperglycemia) results from deficiencies in insulin action, secretion or both, and evidence shows that oxidative stress plays a major role in the pathogenesis of DM and its devastating complications. Typically, hyperglycemia is associated with increased oxidative stress, and contributes to diminished insulin secretion and action. Diabetic patients have diminished antioxidants, which further increase oxidative stress and development of chronic diabetic wounds. Chronic diabetic wounds exhibit a significant influence on debility, ill health, and death [5], and the reduction of ROS levels and oxidative stress induced damage through antioxidative methods improves chronic diabetic wound healing [6]. In the progression of wound healing, apoptosis is essential in eradicating unwanted cells and advances the development of granulation tissue. Apoptosis is an involuntary cell death, enabling a well-coordinated development and removal of cells in wounded and infected tissue, and in DM excessive apoptosis is one of the factors that causes wound chronicity [7,8]. Typically, cellular apoptosis is characterised by morphological changes including shrinkage, condensation of chromatin, blebbing of the cell membrane, and formation of apoptotic bodies with no inflammation [9]. Involuntary cell death is a multifaceted process that can either be induced by intracellular or extracellular apoptotic signalling in reaction to a stress, bringing about cell suicide. Cell suicide follows specific overlapping steps of stimulation, recognition, effectors, and elimination. In chronic diabetic wounds, oxidative stress decreases the appearance of antiapoptotic Bcl 2 proteins, and increases the appearance of proapoptotic Caspases, FS-7-associated surface antigen (FAS), and Bcl 2-associated X (BAX) proteins. Caspases are critical players in cellular apoptosis, and all known caspases have three domains consisting of a small subunit, a large subunit, and an amino terminus. Caspase 3 effects and executes cell apoptosis by coordinating DNA degradation [7].

PBM is a process that is initiated by low-powered light from lasers or LEDs that interacts with chromophores present within the cells/tissue to stimulate or inhibit biological activities. The effect of PBM is influenced by the wavelength, power, fluence and interval of application. The mitochondrial cytochrome c oxidase absorbs the light energy and converts it into photochemical energy resulting in increased adenosine triphosphate (ATP) production, protein synthesis and cell proliferation. PBM has anesthetic, anti-inflammatory, and stimulatory effects in wound healing and tissue regeneration, and in chronic diabetic wounds reduces oxidative and nitrosative stress [10,11]. Ayuk *et al*., [12] noted an increase in cell viability and proliferation when normal, wounded, diabetic wounded, hypoxic wounded, and diabetic hypoxic wounded cells were irradiated at a wavelength of 660 nm with a fluence of 5 J/cm^2 *in vitro*. The aim of this study was to assess the effect of PBM at a wavelength of 660 nm and a fluence of 5 J/cm^2 on stress-induced apoptosis in diabetic wounded fibroblast cells *in vitro*.

2. Methodology

American Type Culture Collection WS1 human skin fibroblast cells (ATCC®, CRL-1502™) were cultivated using standard culture procedures. Two models were used in the study, namely diabetic (D) and diabetic wounded (DW). An *in vitro* diabetic model was achieved by continuously cultivating WS1 cells in supplemented minimum essential medium (MEM) containing an additional 17 mM D-glucose, thereby mimicking a hyperglycemic condition [13]. To perform experiments, cells (6 X 10^5) were cultivated in 3.4 cm diameter tissue culture plates and incubated at 37°C in 5% CO_2 for attachment. After 24 h, a central scratch was performed 30 min pre-irradiation in the wounded cell model thereby creating a cell free zone adjoined by cells on both sides of the “wound” in the confluent monolayer [14]. Cell culture plates, with the lids off, were exposed to laser light from above in the dark. Table 1 shows the laser parameters used in this study. To treat cells with PBM, a 660 nm diode laser at an energy density (fluence) of 5 J/cm^2 was used, and after irradiation cells were incubated for 48 h. Cells were then analysed for viability using the trypan blue negative staining assay, apoptosis using the Caspase-Glo® 3/7 assay (Promega, PRG8090), and qualitative immunofluorescence

microscopy using anti-caspase 3 antibody (Sigma-Aldrich; C8487), and a secondary anti-rabbit fluorochrome tagged antibody (Sigma-Aldrich; 12-507). 4′,6-Diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. Experiments were repeated three times (n=3), and for the Caspase-Glo® 3/7 assay tests were done in duplicate, the average of which was used. SigmaPlot version 14 (Systat Software, Inc.) was used for statistical analysis. Statistical difference between groups was distinguished by using the Student *t* test. Analysis of variance (ANOVA) followed by Dunnett's test was used to compare differences between D and DW cell models. Cohens d was used to determine the effect size. Results are shown as standard error of the mean (SEM) and statistical significance is shown in the graphs as *$p<0.05$ and **$p<0.01$.

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

3. Results

In this study, PBM at a wavelength of 660 nm with a fluence of 5 J/cm^2 significantly increased cellular viability in PBM treated DW cells ($p<0.05$) when compared to their control cells at 48 h (Figure 1). However, the increase in cell viability observed in PBM treated D cell models was not significant ($p=0.07$). When compared to untreated D cells, a significant decrease was noted in untreated DW cells ($p<0.05$), and a significant increase in PBM treated DW cells ($p<0.05$). A significant decrease in cellular viability was noted in untreated DW cells ($p<0.01$) when compared to PBM treated D cells. No significant difference was noted between PBM treated DW and D cells ($p=0.357$). Caspase-3/7 mediates the advancement of cell apoptosis, and in this study, there was a significant decrease in apoptosis in PBM treated DW cells ($p<0.01$) when compared to their control cells 48 h after irradiation (Figure 2). However, the decrease observed in PBM treated D cell models was not significant when compared to their untreated D cells ($p=0.09$). When compared to untreated D cells, there was a significant increase in apoptosis in untreated DW cells ($p<0.05$). However, a significant decrease was noted in PBM treated DW cells ($p<0.05$). A significant increase in apoptosis was noted in untreated DW cells ($p<0.05$) when compared to PBM treated D cells. As was seen in cell viability, no significant difference in apoptosis was noted between PBM treated D and DW cells ($p=0.133$). To verify apoptosis in untreated and PBM treated cells, qualitative immunofluorescence microscopy was conducted (Figure 3) and showed decreased signal of cytoplasm-localised caspase 3 in both PBM treated D and DW cells when compared to their untreated control cells.

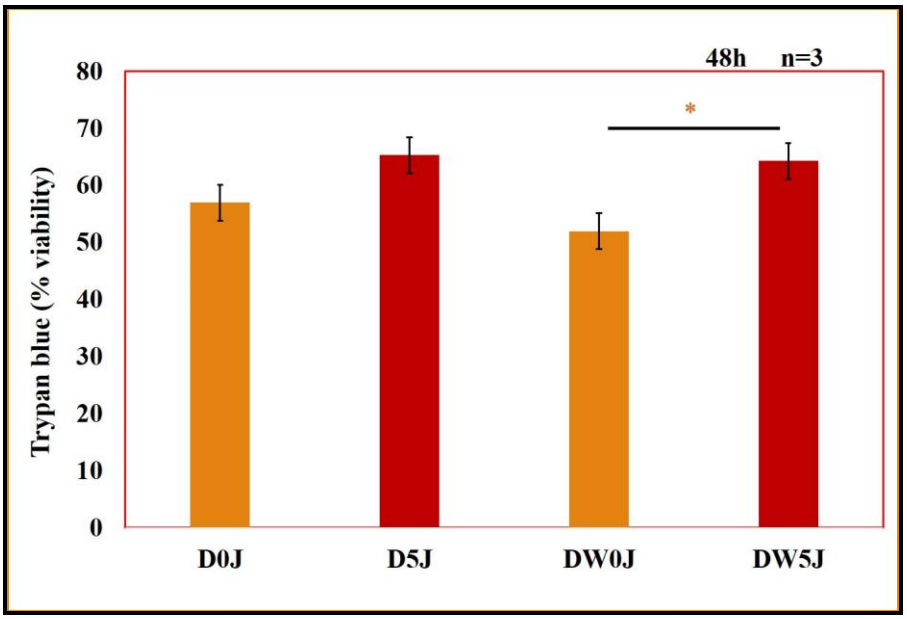

Figure 1. Cell viability was assessed by the trypan blue negative staining assay at 48 h in untreated (0 J/cm^2) and photobiomodulation (PBM) treated (5 J/cm^2) diabetic (D0J; D5J) and diabetic wounded (DW0J; DW5J) cells. Significant probability is shown as *P<0.05 ±SEM.

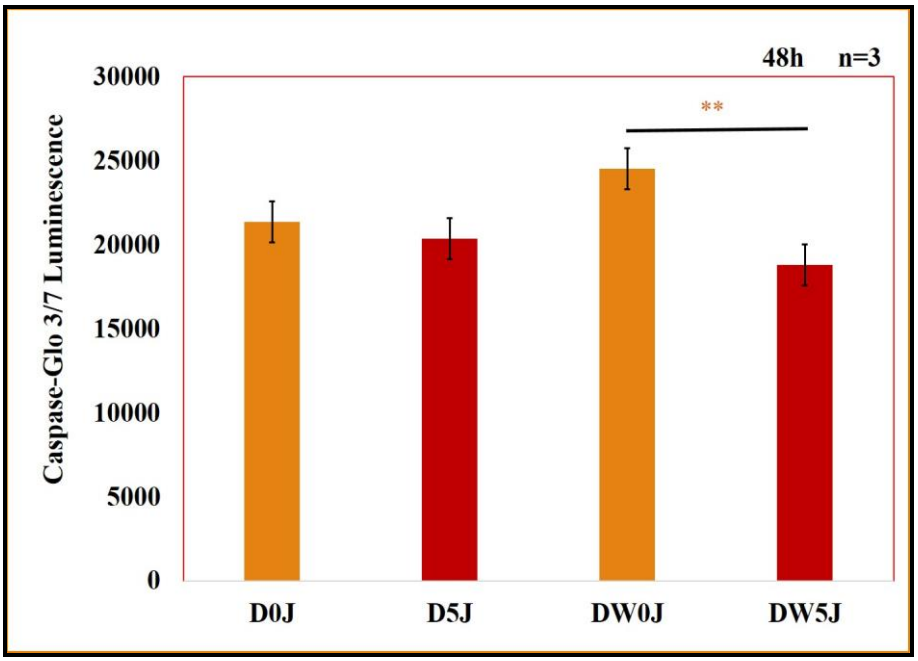

Figure 2. Cellular apoptosis using the Caspase-Glo® 3/7 assay was assessed at 48 h in untreated (0 J/cm^2) and photobiomodulation (PBM) treated (5 J/cm^2) diabetic (D0J; D5J) and diabetic wounded (DW0J; DW5J) cells. Significant probability is shown as **P<0.01 ±SEM.

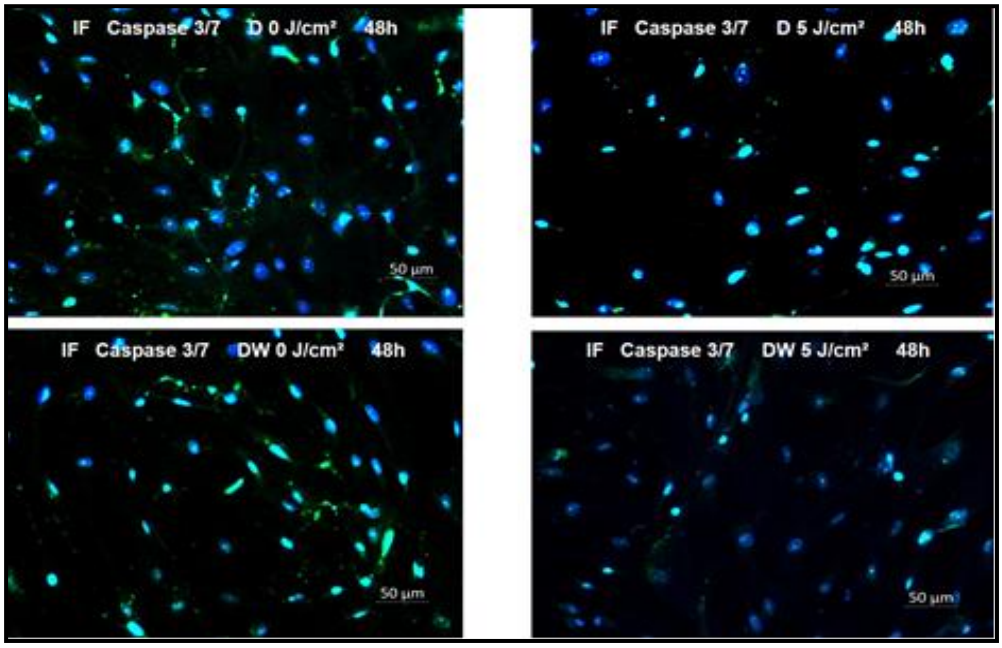

Figure 3. Caspase 3 was determined by immunofluorescence (IF) 48 h post-photobiomodulation (PBM) in untreated (0 J/cm^2) and PBM treated (5 J/cm^2) diabetic (D) and diabetic wounded (DW) cells. The shiny green signal in the micrographs shows caspase-3 activity. 4′,6-Diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei (Blue). (n=3).

4. Discussion and Conclusion

Among key procedures for the physical development in humans is apoptosis. Defective apoptosis has been linked to a variety of diseases including DM and the development of chronic wounds. Hyperglycemia affects the mitochondrial electron transport chain, resulting in increased ROS and the creation of intracellular oxidative stress. The increased ROS production and creation of oxidative stress are crucial influencers of apoptosis and wound chronicity. DM is fast increasing in prevalence and results in considerable economic loss [15,16]. Chronic diabetic wounds and mitochondrial damage increases the release of pro-apoptotic caspases and membrane proteins of the death receptor family (BAX and FAS), and reduces the expression of anti-apoptotic Bcl 2 proteins, resulting in increased cellular apoptosis. Cytosolic caspase 3/7 plays a critical role in facilitating the progress of apoptosis, and the development of treatment methods that target cell apoptosis is critical in the management of chronic diabetic wounds [17].

PBM is a niche area of phototherapy and biophotonics. This treatment method possesses advantages over current treatment methods because of its simplicity to use, increased efficacy, and reduced cost, and its ability to reduce oxidative and nitrosative stress in chronic diabetic wounds [10,11]. Unclear protective mechanisms and treatment parameters makes the use of PBM and standardisation of protocols challenging for its application in chromic diabetic wound healing. In the present *in vitro* study, PBM at 660 nm with 5 J/cm² effectively regulated cellular viability and apoptosis in diabetic cells and diabetic cells that were wounded. There was an increase in cell viability in both wounded and non-wounded PBM treated diabetic cells. Diabetic wounded cells are more stressed, and comparing cell viability of control diabetic wounded cells to less stressed control diabetic cells revealed a significant reduction in viability, indicating the effect of high sugar concentration and wounding on cell viability. However, when diabetic wounded cells were irradiated at 660 nm there was a significant increase in viability compared to non-treated diabetic cells, indicating a positive effect of PBM on cell viability in diabetic wounded cells. This observation was confirmed when diabetic wounded PBM treated cells were compared to PBM treated diabetic cells that revealed no

significant difference. The same observation was noted in cellular apoptosis. There was a reduction in apoptosis in both diabetic and diabetic wounded PBM treated cells compared to their control cells. In all these findings, the effect size using cohens d was moderate, indicating that at 660 nm with 5 J/cm², PBM has a moderate effect on wound healing by increasing cellular viability and reducing cellular apoptosis, a possible molecular mechanism of PBM in quickening diabetic wound healing *in vivo*. These findings signify an effectual therapeutic approach for quickened healing of chronic diabetic wounds. Nevertheless, further studies including clinical, are needed to find out more about the effect of PBM at 660 nm with 5 J/cm² on cellular phenotypic variations at a molecular level.

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