Photobiomodulation at 830 nm modulates proliferation and migration of wounded fibroblast cells

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Abstract. Wound healing is a complex and dynamic process that involves restoring damaged tissue structure and function. Delayed wound healing often advances to chronic non-healing wounds due to reduced cellular proliferation and migration. Photobiomodulation (PBM) involves the application of low-powered light typically in the visible red and near-infrared (NIR) spectrum to modulate cellular mechanisms and has been shown to speed up healing *in vivo*. However, the underlying mechanisms are not well understood. This study aims to determine the effect of PBM using NIR light at 830 nm with 5 J/cm^2 on the proliferation and migration of wounded human fibroblasts. Commercially acquired human fibroblast cells (BJ-5ta, ATCC® CRL-4001™) were utilized, and two cell models, namely, normal and wounded (central scratch assay), were designed. Cell models were incubated for 24 and 48 h post-irradiation, followed by different investigational tests for cellular morphology and migration rate (inverted microscopy), and proliferation (BrdU, flow cytometry). PBM at 830 nm with 5 J/cm^2 modulates cell proliferation and migration and may aid in the enhanced wound repair process observed *in vivo*.

1. Introduction

The prevalence of chronic wounds is rising at a perturbing rate due to populations undergoing industrialization, aging, and becoming more sedentary, and pose a fundamental global economic public health burden. Wounds arise as a result of traumatic injuries, including abrasions and lacerations. Ordinarily, wounds heal in a timely (within a few days/weeks, typically in 3 months) and orderly frame; however, due to underlying pathologies such as keloids, uremia, fibrosis, jaundice, and hereditary healing disorders, some wounds fail to do so and develop into chronic wounds. Wound healing involves a systematized integration of biological and molecular systems that induce a predictable progression of tissue repair. Wound healing is orchestrated via four fundamental and spatially overlapping cascades: hemostasis, inflammation, proliferation, and maturation. For optimum healing of cutaneous wounds, fibroblasts, immune surveillance cells, platelets, keratinocytes, and microvascular cells play vital roles in restoring damaged tissue structure and function and reinstating tissue integrity [1]. Fibroblasts are the primary reconstructing cells involved in critical processes such as breaking down the fibrin clot, constricting the wound, secreting and depositing new extracellular matrix (ECM) proteins, and providing collagen and granulation tissue. They are responsible for secreting essential cytokines and growth factors for cell proliferation and differentiation conducive to wound repair [2]. Impaired fibroblast functionality in diabetic patients often leads to decreased collagen synthesis and wound strength. According to You *et al.* [3], chronic wounds are onerous to treat due to their diminished response to conventional treatments.

Photobiomodulation (PBM) employs specific wavelengths of visible red and near-infrared (NIR) light to expiate specific cellular and tissue impairments. This therapeutic technique uses light with distinctive properties of coherence (light waves in temporal and spatial phases), collimation (rays are parallel and travel in a single direction without divergence), and monochromaticity (single wavelength radiation) [4], enabling non-invasive penetration of the skin surface. PBM is a non-thermal therapy that involves the application of low-powered light sources, such as lasers, light-emitting diodes (LEDs), and broadband light using appropriate filters, in the visible light (400- 800 nm) and infrared (760- 1400 nm) spectrum to promote tissue regeneration [4,5]. Several studies have demonstrated PBM as a promising therapeutic method that elicits healing [6] and advances the proliferation phase of wound healing [7].

Additionally, studies have demonstrated different physiological effects when comparing PBM in the visible red light and NIR spectrum, indicating that PBM has biostimulatory effects in a dose and wavelength-dependent manner [8,9]. An optimal dosage of PBM evokes cellular activity, including cell proliferation, viability, migration, and growth factor production, which enhances the wound repair process in non-healing chronic wounds. Based on the optical window model, NIR light has been reported to penetrate tissues deeper than red light, which is ascribed to the reduced absorption by hemoglobin and melanin [10]. Despite NIR light having a deeper penetration into the tissue, most *in vitro* studies use visible red light, leaving a knowledge gap in the NIR range. Despite global interest in PBM therapy, the application of this therapy has not been acclimatized or routinely practiced in South Africa.

This study aimed to determine the effects of PBM at 830 nm on the proliferation and migration of fibroblast cells, which are principal contributing aspects to wound healing.

2. Methodology

Commercially purchased human fibroblast cells (BJ-5ta, ATCC® CRL-4001™) were utilized and cultured under standard guidelines. Two cell models were designed: normal (N) and wounded (W). Briefly, cells were incessantly grown in minimum essential media (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% amphotericin B, 1% penicillin-streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), and 1 mM sodium pyruvate. Cells were then rinsed twice with pre-warmed Hank's Balanced Salt Solution (HBSS) and subsequently detached with 1 mL/25 cm^2 of TrypLE™ Express. Cell quantification was conducted, and cells were seeded at a density of 6×10^5 (morphology and migration rate) or 1×10^6 for flow cytometry (proliferation assay) into 3.4 cm diameter cell culture dishes in 3 mL supplemented MEM and were incubated at 37°C (5% carbon dioxide (CO_2); 85% humidity) overnight to facilitate cellular attachment.

Post-incubation, the culture media was discarded, and cells were rinsed twice with 1 mL HBSS and replaced with fresh media. To achieve a wounded cell model (W), a sterile 1 mL disposable pipette was used to create a central scratch (CS), or "wound," in a confluent cell monolayer [11]. The CS method is based on the observation that upon creating a cell-free zone across the center of the cell monolayer, the cells on the "wound" margins will migrate towards the center to close the created gap [12]. Post-wounding, the cells were incubated for 30 min to allow them to settle [11]. Cells were irradiated with a continuous wave diode laser at a wavelength of 830 nm, and a fluence of 5 J/cm^2. Laser parameters are summarized in Table 1. Post-irradiation, cells were incubated at 37°C (5% CO_2; 85% humidity) for 24 and 48 h.

Post-irradiation, an inverted light microscope (Olympus CKX41) was used to determine morphological changes in both experimental models, and images were captured using the AnalySISgetIT software at 0 h, 24 h and 48 h to assess wound closure. The distance between the wound margins was measured and used to quantify the rate of cell migration, which was expressed in percentages using the following equation:

$$At_{0h}\text{-}At_{time}/At_{0h}\times 100$$

where At_{0h} is the distance between wound margins of the central scratch at 0 h, and At_{time} is the successive distance between the wound margins of the central scratch at various time points [13].

Flow cytometry using the BD Pharmingen fluorescein isothiocyanate (FITC) 5-Bromo-2'-deoxyuridine (BrdU) Flow Kit (The Scientific Group, BD Sciences, 559619/557891) was used to

evaluate cell proliferation. BrdU, an analog of thymidine, a DNA precursor, is integrated into newly synthesized DNA in cells acceding and advancing through the S phase of DNA replication (DNA synthesis). In this procedure, the quantification and categorization of cells actively synthesizing DNA, as related to their cell cycle position (i.e., S phase) explicated by their 7-ADD staining intensities, is accomplished by a two-color cytometric analysis. The test was conducted using the manufacturer's instructions. Unstained cells were used to establish easy visualization and gating of cells on forward scatter (FSC) and side scatter (SSC) plots.

Samples were analyzed three times (n=3), and statistical analysis was done using SigmaPlot version 14.0 (SYSTAT). Differences between groups were determined using the Student t-test and One-Way Analysis of Variance (ANOVA) for each independent variable and were considered statistically significant when $p< 0.05$.

Table 1. Laser irradiation parameters.

Variables	
Light source	Diode laser
Wavelength (nm)	830
Emission	Continuous wave
Power output (mW)	114
Spot size (cm^2)	9.1
Power density (mW/cm^2)	12.56
Irradiation exposure (s)	364
Fluence (J/cm^2)	5
Energy (J)	41.5

3. Results and Discussion

Chronic or non-healing wounds fail to progress through the wound healing phases in a timely reparation process primarily due to impaired wound oxygenation, infections, venous insufficiency, and diseases that interrupt the normal physiological condition, predisposing it to impaired wound healing. PBM imparts an array of therapeutic interventions, such as activating different intracellular signaling pathways involved in cellular proliferation, migration, and wound healing [14]. Optimal use of PBM augments cell viability and the rate of proliferation in various cell types, including but not limited to fibroblasts [15], mesenchymal stem cells [16], and epithelial cells [17]. Hehenberger and colleagues [18], suggested that fibroblasts from chronic wounds demonstrate a decreased proliferation rate. Therefore, therapeutic methods inciting increased fibroblast activity are essential for advancing wound healing.

In this study, microscopically, fibroblast cells displayed the typical elongated and spindle shape, with multi-polar projections (Figure 1). In W irradiated (5 J/cm^2) cell models, the findings demonstrated increased haptotaxis (an outgrowth of cells along the wound margins) and chemotaxis (movement of cells towards the central scratch) when compared to the W non-irradiated (0 J/cm^2) controls.

W irradiated (5 J/cm^2) cells exhibited a significant increase ($p<0.05$) in migration rate at 24 h, suggesting that PBM effectively induces chemotaxis and haptotaxis, and accelerates fibroblast cell migration in wound healing. However, at 48 h, the increase in the rate of cell migration observed in W irradiated (5 J/cm^2) cells was not statistically significant ($p=0.943$) when compared to their non-irradiated (0 J/cm^2) control cells (Figure 2). These results correspond with Ayuk and colleagues' [19] study using diabetic wounded fibroblast cells (WS1). The fibroblasts exhibited evidence of haptotaxis and chemotaxis when irradiated at 660 nm (5 J/cm^2). In this study, W irradiated (5 J/cm^2) cells migrated faster, which is verified by the significant increase in migration rate at 24 h. These results are supportive of the incomplete wound closure observed in W irradiated (5 J/cm^2) cells, which contradicts the results when the fibroblast cells are irradiated at a red wavelength of 632 nm [8] and 660 nm [20]. Additionally, comparing W cell models at 24 h with their respective models at 48 h demonstrated a statistical difference ($p<0.01$).

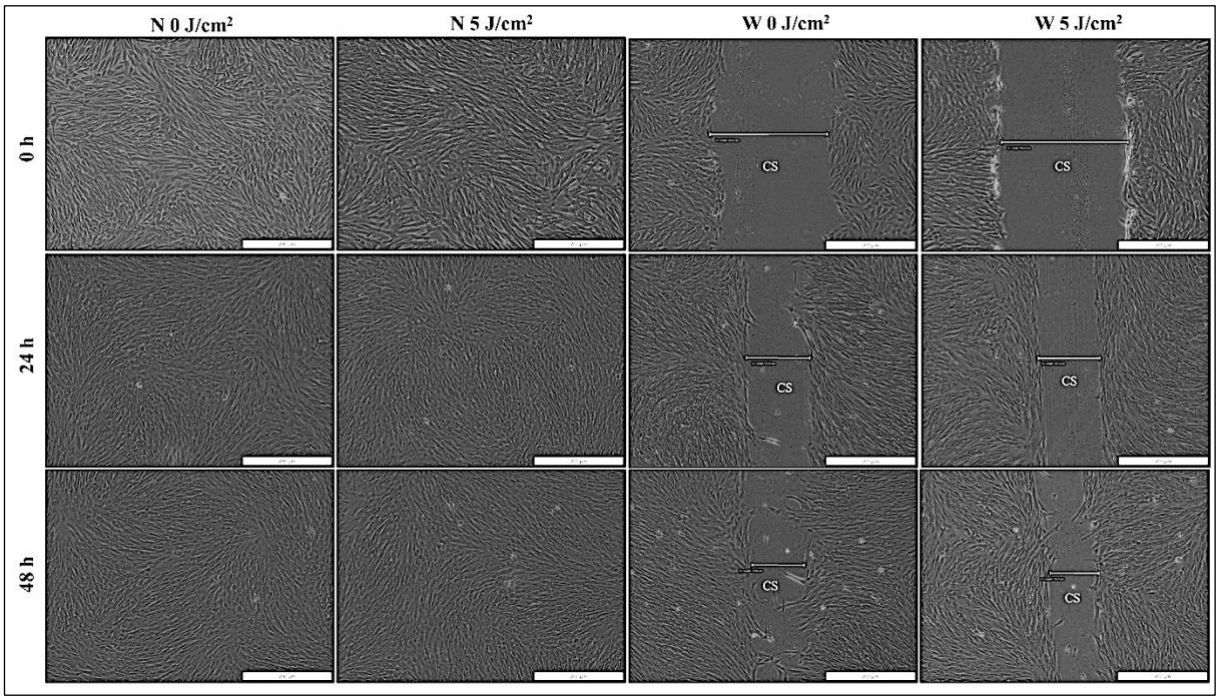

Figure 1. Micrographs displaying the morphology of normal (N) and wounded (W) cell models *in vitro* at 0 h, 24 h and 48 h post-irradiation. Cells retained their well-known elongated and spindle-shaped fibroblast morphology. At the same time, cells in the W model demonstrated a change in direction and migrated towards the central scratch (CS) to enable wound closure. Scale bar 200 µm (4x magnification).

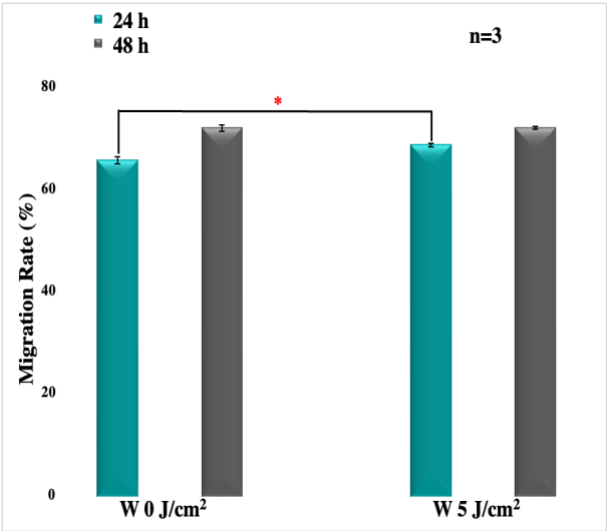

Figure 2. Percentage (%) migration rate in wounded (W) cell models at 24 h and 48 h post-irradiation. W irradiated (5 J/cm^2) cells at 24 h demonstrated a significant increase in migration rate when compared to their non-irradiated (0 J/cm^2) controls. Statistical significance is presented as *p<0.05 (±SEM).

At 24 h post-irradiation, N irradiated (5 J/cm^2) cells demonstrated a significant decrease in cell proliferation (p<0.01), while W irradiated (5 J/cm^2) cells exhibited a significant increase (p<0.05) when compared to their control cells (Figure 3). At 48 h post-irradiation, N irradiated (5 J/cm^2) cells exhibited a non-significant increase (p=0.067) in cell proliferation when compared to their non-irradiated (0 J/cm^2) controls. W irradiated (5 J/cm^2) cells exhibited a significant increase (p<0.05) in cell proliferation compared to their non-irradiated (0 J/cm^2) controls. Thus, demonstrating a significant acceleration in

cellular proliferation in W irradiated cell models as revealed by BrdU staining for cells acceding and advancing through the S-phase of DNA replication. N irradiated (5 J/cm^2) cells displayed no propitious effect of PBM, signifying that PBM therapy is more beneficial for stressed cells and/or cells with impaired growth. Therefore, PBM alters the physiological state of stressed cells, restoring tissue homeostasis and healing responses [20]. On the contrary, Pogrel and colleagues [21], reported that Ga-Al-As lasers at 830 nm and a fluence that ranged from 0.12 to 4.24 J/cm^2 could not augment the proliferation of keratinocytes and fibroblasts. A study by Almeida-Lopes *et al.* [15], demonstrated that PBM in the NIR spectrum (780 nm; 2 J/cm^2) increased cell proliferation of human gingival fibroblasts.

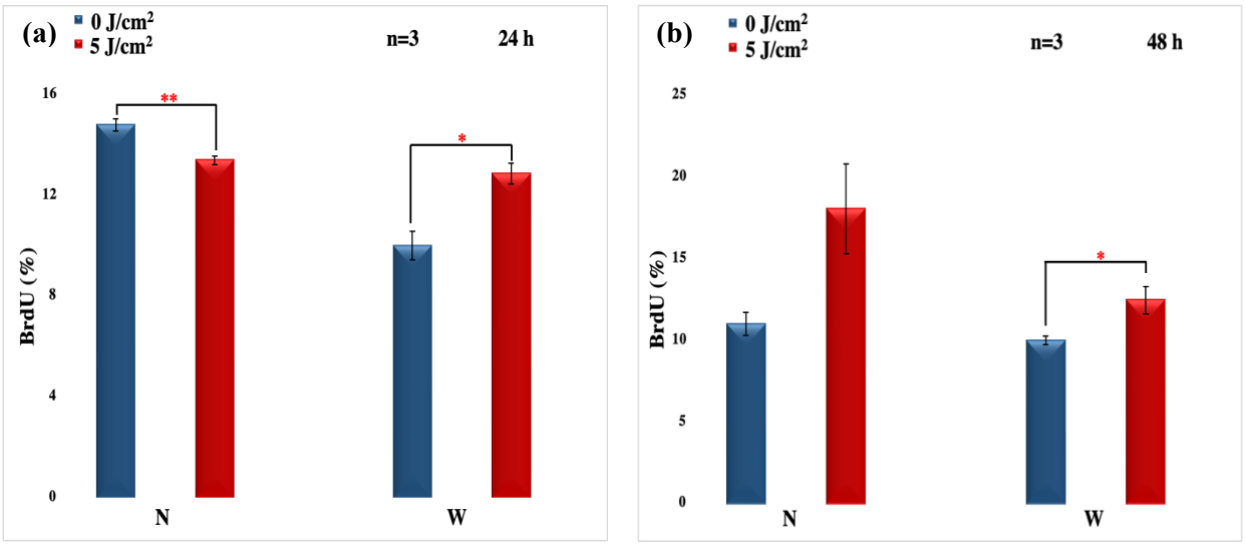

Figure 3. Percentage of cells in proliferative S- (DNA synthesis) phase as measured by flow cytometry in normal (N) and wounded (W) cell models **(a)** 24 h, and **(b)** 48 h post-irradiation. Statistical significance is presented as *p<0.05 and **p<0.01 (±SEM).

4. Conclusion

In conclusion, the present study's findings suggest that PBM at 830 nm using 5 J/cm^2 has a photobiostimulatory effect on cellular migration and proliferation and has the potential to augment wound healing. However, no complete wound closure was detected at 48 h, which contradicts the results when fibroblast cells are irradiated at a red wavelength of 660 nm. These findings suggest that although there is a significant improvement in cell migration and wound closure at 830 nm, PBM of cells *in vitro* in the visible red region appears to be more effective and pronounced. To verify these discrepancies, more comparative research on PBM at 660 nm and 830 nm should be conducted in both *in vitro* and *in vivo* studies.

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