Nuclear translocation of Map Kinase and release of basic fibroblast growth factor following photobiomodulation at 660 nm in diabetic wounded cells

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Abstract. Mitogen-activated protein kinase (MAPK) signalling is one of the best characterised signal transduction pathways in cell biology and is involved in wound healing processes. Photobiomodulation (PBM) has been used to induce physiological changes and has been shown to improve wound healing processes, however the underlying molecular and cellular mechanisms of action remain largely unexplained. The purpose of this study was to determine the effect of PBM at 660 nm on nuclear translocation of MAPK and release of basic fibroblast growth factor (bFGF) in diabetic wounded fibroblast cells *in vitro*. This was evaluated by irradiating cells at a wavelength of 660 nm with 5 J/cm^2 and incubating them for 24 and 48 h. Non-irradiated cells (0 J/cm^2) served as controls. b-FGF was measured by the Enzyme Linked Immunosorbent Assay (ELISA) and translocation of phosphorylated MAPK was assessed by immunofluorescence. PBM of diabetic wounded cells showed an increased release of bFGF and translocation of MAPK in irradiated cells at 24 and 48 h as compared to non-irradiated cells. The findings of this study showed that PBM is capable of inducing the release of bFGF and activation of MAPK in diabetic wound cells *in vitro*, thus facilitating wound healing under diabetic conditions.

1. Introduction

Diabetes Mellitus (DM) is a chronic metabolic disease characterised by hyperglycaemia due to the absence or insufficient production of insulin, or an inability of the body to properly use insulin (insulin resistance). According to the International Diabetes Federation (IDF), DM is a global health burden with 463 million cases seen in adults between the ages of 20 to 79 (9.3%). South Africa has a prevalence rate of 12.7% [1]. Diabetic complications are due to high levels of glucose and protein glycation. Major complications include angiopathy, neuropathy, cardiovascular diseases, and retinopathy. Approximately 15% of diabetic patients experience lower limb complications, including chronic non-healing ulcers, largely due to neuropathy and angiopathy [2]. These ulcers commonly necessitate lower extremity amputation, often resulting in a poor-quality of life and the untimely death of the patient [3]. Globally, it has been estimated that every 30 seconds a diabetic patient loses a limb or part thereof due to amputation [1].

The normal wound healing process involves four sequential biological overlapping phases namely, homeostasis, inflammation, reepithelization, and tissue remodelling. The expected timeframe for

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successful wound healing is three months, however once this time is exceeded due to conditions such as diabetes, the wound is considered to be chronic [4]. This delay in the wound healing process has been linked to a reduction in cellular processes due to a decrease in cell migration and proliferation, as well as a lack of growth factor and collagen synthesis [5].

The closure and repair process of wounds following tissue damage employs a series of molecular and cellular activities that aim to restore the injured tissue. The regulation and redirection of the wound healing process is mainly achieved by the presence of growth factors and cytokines [6]. It is well known that the activities of growth factors are deficient in diabetic wounds because of a reduction in their production and/or an increased rate of their elimination. Fibroblast growth factor (FGF) is one such growth factor, which has considerable effects on tissue repair and regeneration. There are 22 members of the FGF family, and basic FGF (bFGF or FGF2) has been shown to regulate cell proliferation, migration, and differentiation [7]. FGF interacts with their corresponding receptor, FGFR in the presence of heparan sulphate (HS) as a cofactor. This binding triggers the formation of the ternary FGF-FGFR-HS complex which in turn activates the FGFR intracellular kinase domain through the phosphorylation of a tyrosine residue. The activated domain is coupled to intracellular signalling pathways, including the Ras/ mitogen-activated protein kinase (MAPK) pathway [6]. MAPK is one of the best characterised signal transduction pathways, and an important bridge in cell biology that plays a crucial role in transducing signals from the extracellular milieu to the cell nucleus where specific genes are activated for cell growth, division, and differentiation that leads to improved wound healing [6].

Photobiomodulation (PBM) utilises non-ionised, low powered light sources (including lasers and light emitting diodes) with wavelengths typically in the visible red or near infrared (NIR) spectrum to promote physiological changes and render therapeutic benefits without causing damage [8,9]. The principle of PBM is based on the absorption of light by a chromophore which stimulates the biological system in an organism to speed up and activate an array of cellular processes that results in downstream physiological effects facilitating the process of wound healing, and concomitantly reducing pain, inflammation, and oedema [9,10]. Studies have shown that PBM at 660 nm increases the production of growth factors and the expression of genes that play an important role in wound healing [11-13]. PBM has also been used to treat a variety of conditions and has been found to be beneficial in the treatment of diabetic wounds [5, 9]. This study aimed to determine the effect of PBM at 660 nm on the nuclear translocation of MAPK and release of bFGF in diabetic wounded cells *in vitro*.

2. Materials and methods

This study used commercially purchased human skin fibroblast cells (WS1, ATTC® CRL-1502™). Ethical clearance was obtained from the University of Johannesburg, Faculty of Healthy Sciences Research Ethics Committee (REC-487-2020). A diabetic cell model was achieved by continuously growing cells in minimum essential media (MEM) containing an additional 17 mM D-glucose (MEM already contains glucose at a basal concentration of 5.6 mM, thus cells are grown in 22.6 mM D-glucose) [13-15]. Cells were seeded in 3.4 cm diameter tissue culture plates (6×10^5) and incubated overnight. A wound was created via the central scratch assay using a sterile 1 mL pipette to create a cell-free zone in the confluent monolayer of cells. After creating the cell-free zone, cells were incubated for 30 min before irradiation in order to allow them to adapt [14]. The effect of PBM on nuclear translocation of MAPK and release of bFGF in a diabetic wounded cell model was evaluated by irradiating cells at a wavelength of 660 nm with a fluence of 5 J/cm^2 (table 1), while non-irradiated (0 J/cm^2) cells served as the control. A fluence of 5 J/cm^2 has been used extensively by the research group *in vitro* as an optimal fluence at a wavelength of 660 nm [12,14]. Cells were irradiated in the dark to eliminate the interference of surrounding light. The laser light was directed to the cells via fibre optics from above with the culture dish lid off in 1 mL of culture media.

Nuclear translocation of ERK1/2(MAPK) was detected by immunofluorescence whereby cells that were grown on coverslips placed in 3.4 cm diameter tissue culture plates were fixed with 4% paraformaldehyde. Following 15 min of incubation at room temperature cells were permeabilised with 0.1% Triton-X 100 in phosphate buffered saline (PBS). Thereafter, non-specific antibody binding was

blocked in 1% Bovine Serum Albumin (BSA) in PBS. MAPK was first labelled with 1:250 primary antibody (anti-Map kinase, activated (diphosphorylated ERK-1&2) antibody, mouse monoclonal; Sigma Aldrich, M9692). The primary antibody was detected using a 1:100 fluorescently labelled secondary antibody (Goat Anti-mouse IgG, H&L, FITC conjugate, Sigma Aldrich, 12-506). Nuclei were counterstained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI). Slides were observed under a fluorescent microscope (Carl Zeiss, Axio Observer Z1).

Table 1. Laser parameters.

Variables	Diode laser
Wavelength (nm)	660
Fluence (J/cm^2)	5
Power output (mW)	100
Power density (mW/cm^2)	11
Spot size (cm^2)	9.1
Irradiation time	7 min 57 s
Energy (J)	47.7

The release of bFGF into the culture media was analysed using a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit according to the manufacturers' details (Invitrogen, Human FGF basic ELISA Kit, Thermo Fisher Scientific, KHG0021). Absorbance was read at 450 nm using the VICTOR Nivo$^{TM.}$ Multimode Plate Reader (PerkinElmer).

Experiments were conducted three times (n=3). ELISA results were statistically analysed using SigmaPlot version 13.0 (Systat Software, Inc.). Differences between groups was determined using the student t-test and each independent variable was considered statistically significant when $P<0.05$.

3. Results and discussion

Immunofluorescent results in irradiated (5 J/cm^2) diabetic wounded cells showed strong translocation of MAPK as compared to control cells at 24 h (figure 1). At 48 h post-irradiation there was little translocation of MAPK with some localisation around the nucleus.

bFGF ELISA results showed a significant increase in bFGF released into the surrounding media in irradiated cells (5 J/cm^2) at 24 h ($P < 0.05$) (figure 2), while at 48 h there was a significant decrease ($P< 0.01$) (figure 3). Fibroblasts migrate into the wound site and start to release growth factors [16]. The binding of bFGF to its receptor (FGFR) leads to activation of the MAPK signalling pathway, and hence translocation of MAPK. The cells then start to participate in the process of wound healing, such as reducing wound size by contraction. The increase in bFGF at 24 h correlates with the increased MAPK translocation seen in the fluorescent images. The decrease seen in bFGF at 48 h may be due to the consumption of bFGF by the cells through paracrine signalling. The decrease in bFGF at 48 h also corresponds with the decrease seen in MAPK translocation at 48 h.

Other studies using PBM have found an increase in bFGF. A similar study conducted by our research group on fibroblast cells cultured under hyperglycaemic conditions found that PBM at a wavelength of 632.8 and 830 nm irradiated with a fluence of 5 J/cm^2 increased bFGF [13,14]. In another study, human skin fibroblast cells cultured under hyperglycaemic conditions and irradiated at the same wavelength of 632. 8 nm once a day for three days at a fluence of 1 J/cm^2 also showed increased release of bFGF [17]. Human gingival fibroblast cells grown under normal conditions and irradiated at a wavelength of 780 nm and a fluence of 2-3 J/cm^2 showed increased production and secretion of FGF [18]. A wounded rat animal model irradiated at a wavelength of 660 nm and a fluence of 2.4 J/cm^2 showed that PBM

plays a critical role in diabetic wound healing by stimulating the release of essential growth factors (bFGF and FGF) [19].

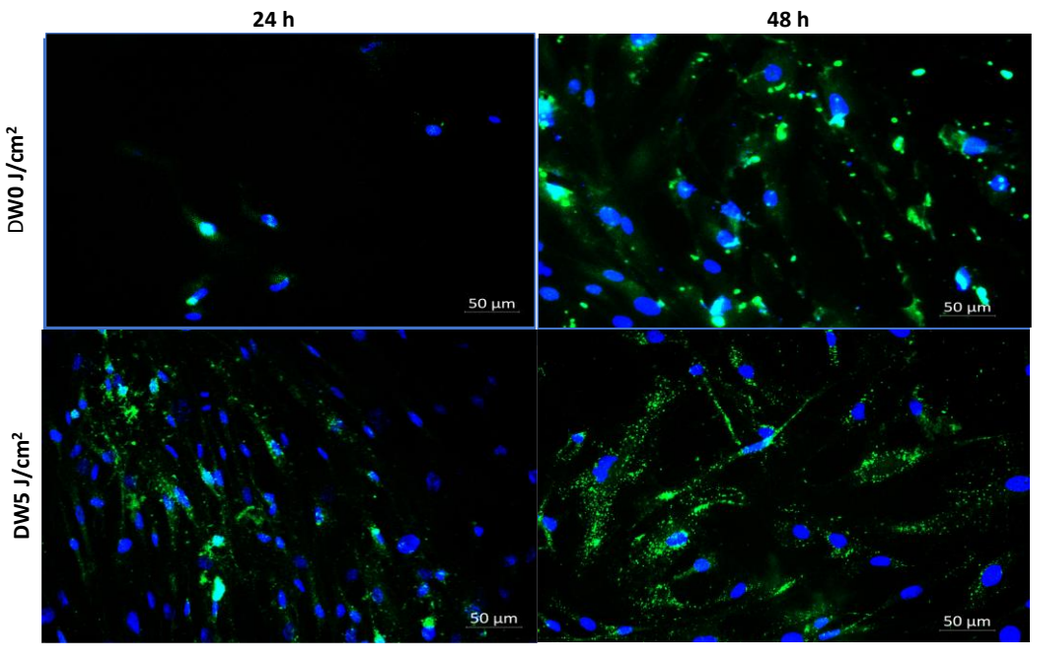

Figure 1. Diabetic wounded (DW) fibroblast cells were irradiated at 660 nm with 5 J/cm^2 and incubated for 24 or 48 h. Control cells were not irradiated (0 J/cm^2). MAPK was fluorescently labelled with FITC (green) and nuclei stained with DAPI (blue). Irradiated cells showed nuclear translocation of MAPK, particularly 24 h post-irradiation (magnification 200x).

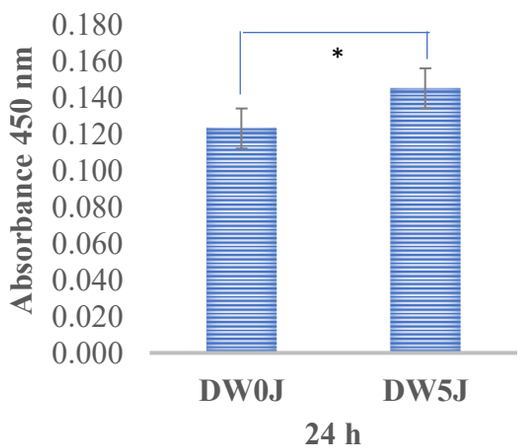

Figure 2. Basic Fibroblast Growth Factor (bFGF) analysed at 24 h in non-irradiated diabetic wounded (DW 0 J/cm^2) control and irradiated diabetic wounded (DW 5 J/cm^2) cells. Statistical significance is shown as *P<0.05, (±SEM).

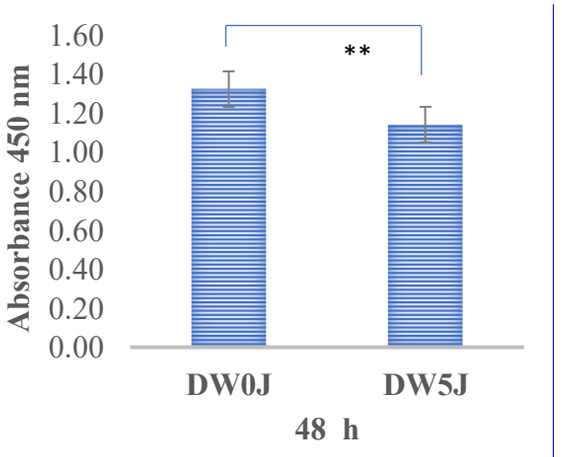

Figure 3. Basic Fibroblast Growth Factor showing bFGF) analysed at 48 h in non-irradiated diabetic wounded (DW 0 J/cm^2) and irradiated diabetic wounded (DW 5 J/cm^2) cells. Statistical significance is shown as **P<0.01, (±SEM).

4. Conclusion

This study aimed to determine the effect of PBM at 660 nm with 5 J/cm^2 on nuclear translocation of MAPK and release of bFGF in diabetic wounded cells. The findings of this study showed that PBM at 660 nm and a fluence of 5 J/cm^2 promoted the release of bFGF *in vitro* at 24 h under hyperglycaemic conditions. PBM promoted nuclear translocation of MAPK, and hence activation of the MAPK signalling pathway. These results may explain the downstream effects seen in response to PBM, including increased proliferation and migration. Further studies into the effects of 660 nm on the MAPK pathway are warranted.

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References

[1] International Diabetes Federation, IDF 2019 *Diabetes Atlas,* 9th edition, available from https://www.diabetesatlas.org/en/ Accessed on 26/04/21
[2] Zhang C, Ponugoti B, Tian C, Xu F, Tarapore R, Batres A, Alsadun S, Lim J, Dong G and Graves D T 2015 *J. Cell. Biol.* **209** 289-303
[3] Järbrink K, Ni G, Sönnergren H, Schmidtchen A, Pang C, Bajpai R and Car J 2017 *Syst. Rev.* **6.** 15
[4] Rajendran N K, Dhilip Kumar S S, Houreld N N and Abrahamse H 2019 *J. Cell. Commun. Signal.* **13** 151- 62
[5] Houreld N N 2014 *Scientific World Journal* **2014** 398412
[6] Ornitz DM and Itoh N 2015 *Wiley Interdiscip. Rev. Dev. Biol.* **4** 215–66
[7] Yun Y-R, Won J E, Jeon E, Lee S, Kang W, Jo H, Jang J-H, Shin U S and Kim H-W 2010 *J. Tissue Eng.* **2010** 218142
[8] Anders J J, Arany P R, Baxter G D and Lanzafame R J 2019 *Photobiomodul. Photomed. Laser Surg.* **37** 63-5
[9] Houreld N N 2019 *Appl. Sci.* **9** 5114
[10] Dompe C et al. 2020 *J. Clin. Med.* **9** 1724
[11] Phillip V P, Chang T Y, Ryan B and Baxter D 2011 *Photomed. Laser Surg.* **29** 285-304
[12] Jere S W, Houreld N N and Abrahamse H 2018 *J. Photochem. Photobiol. B.* **179** 74–83
[13] Houreld N N and Abrahamse H 2008 *Lasers Med. Sci.* **23** 11–8
[14] Houreld N N and Abrahamse H 2010 *Diabetes Technol. Ther.* **12** 971–8
[15] Ayuk S M, Houreld N N and Abrahamse H 2012 *Diabetes Technol. Ther.* **14** 1110–7
[16] Tracy L E, Minasian R A and Caterson E J 2016 *Adv. Wound Care* **5** 119-36
[17] Esmaeelinejad M and Bayat M 2013 *J. Cosmet. Laser Ther.* **15** 310–7
[18] Castro T N S, Martignago C C S, Assis L, de Alexandria F E D, Rocha J C T, Parizotto N A and Tim C R 2020 *Lasers Med. Sci.* **35** 939-47
[19] Damante C A, De Micheli G, Miyagi S P, Feist I S and Marques M M 2009 *Lasers Med. Sci.* **24** 885-91