Gene expression changes in diabetic wound healing as induced by photobiostimulation in vitro

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Abstract. Diabetes Mellitus (DM) is a complex metabolic disorder resulting in hyperglycaemia. Impaired wound healing is a serious complication of diabetes, and is a severe public health problem. Photobiostimulation is a non-invasive form of treatment known to enhance healing of such wounds using low energy lasers. This study aimed to evaluate the role of photobiostimulation at 830 nm on diabetic wounded fibroblast cells in vitro and extracellular matrix (ECM) gene expression. Normal (N-unstressed), normal wounded (NW-stressed) and diabetic wounded (DW-stressed) fibroblasts were incubated for 48 h after irradiation using a continuous wave diode laser at a wavelength of 830 nm with 5 J/cm². Non-irradiated (0 J/cm²) N- and DW-cells were used as controls. The gene expression profile (84 genes) was assessed using an ECM real-time reverse transcription polymerase chain reaction (RT-PCR) array with the appropriate controls included. Sixty one genes were significantly regulated (55 up-regulated and 6 down-regulated) in N-cells; 40 genes (20 up-regulated, and 20 down-regulated) in NW-cells; and 42 genes (9 up-regulated and 33 down-regulated) in DW-cells. Several genes were down-regulated in DW-cells as compared to N- and NW-cells. Photobiostimulation modulated the expression of important genes in wound healing, including cell adhesion molecules, integrins, ECM proteins, proteases, and inhibitors involved in the ECM. An in depth comprehension of the molecular and biological processes may create an improved therapeutic protocol for the treatment of diabetic wounds.

1. Introduction
Complications of diabetes and other non-healing ulcers remain a major concern in public health. Global statistics show that millions of people suffer from this disease, with a prevalence of 15% with diabetic foot ulcers and 3% with lower limb amputation [1]. Wound healing involves a series of biological processes that must occur appropriately within a given time, and necessitates the interaction of various cells and growth factors to ensure proper healing [2-4]. Fibroblasts are very important in wound healing; they maintain homeostatic balance, and through cell proliferation, differentiation and extracellular matrix (ECM) development stimulates the process of tissue repair by synthesising collagen around the matrix [5,6]. Chronic ulcers may result from an imbalance between ECM formation and degradation [7]. Hyperglycaemia can affect these cellular and biological activities through oxidative stress or alkaline
glycation end (AGE) products which in turn affect changes in genes encoding for ECM proteins and proteases [8], leading to impaired wound healing [9]. Several investigations have shown diabetes to affect gene expression as well as wound healing [10,11]. Photobiostimulation at various wavelengths and dosages is known for its stimulatory effect, and enhances wound healing in animal and clinical studies, as well as cell cultures [12-14]. Investigations have also shown that the interaction of photobiostimulation in cell cultures and animals enhances gene expression [15-17]. For these non-invasive processes to be achieved, wavelengths within the visible and Near Infra-Red (NIR) spectrum are implemented during treatment with a power output of 10-200 mW. This treatment is acceptable in many clinical practices however disputes on protocol and treatment specifications, for different models, create room for more research [13]. The purpose of this study is to evaluate the role of photobiostimulation at 830 nm on diabetic wounded fibroblasts and gene expression.

2. Materials and methods

2.1. Cell Culture and Laser Irradiation

Isolated human skin fibroblasts (Academic ethics Committee, Clearance Reference Number: 01/06, University of Johannesburg) were used to create various models, namely normal (N-unstressed), normal wound (NW-stressed) and diabetic wounded (DW-stressed), (Figure 1A). Cells were cultured via standardised protocols [18]. Approximately 6 x 10^5 cells was re-suspended in growth media and seeded in 3.4 cm diameter culture dishes. Using media with a basal concentration of 5.6 mM/L D-glucose, an <i>in vitro</i> diabetic model was established by adding 17 mM/L D-glucose [19]. The Council for Scientific and Industrial Research (CSIR) / National Laser Center (NLC) of South Africa provided the lasers. Prior to laser irradiation at 830 nm (Fremont, California, USA, RGBlase, TECIRL-70G-830SMA or FC-830-300-mm2-sma-1-0) (continuous wave; 98 mW; 9.1 cm^2; 10.76 mW/cm^2; 5 J/cm <sup>2</sup>; duration of irradiation 7 min 43 s), the stressed wounded models were created by introducing a central scratch on a confluent monolayer of cells using a 1 ml sterile disposable pipette (Figure 1B) [20,21]. Non-irradiated (0 J/cm <sup>2</sup>) N- and DW-cells were used as controls. Post irradiation cells were incubated for 48 h. Total RNA was isolated and quantified, and 1 µg was reverse transcribed into cDNA and the gene profile was determined making use of the human ECM and adhesion molecules RT² Profiler™ real-time reverse transcriptase polymerase chain reaction (RT-PCR) Array (SA Biosciences, PAHS-0132Z) [15]. The statistical analysis was achieved on three repeats of each sample.

![Figure 1A. Micrograph of unstressed fibroblasts seeded to 70-80% confluency.](image1A.png)

![Figure 1B. Stressed fibroblasts created with central scratch (CS).](image1B.png)
3. Results

The results showed that laser photobiostimulation mediated the expression of 84 genes. Sixty one genes were significantly regulated (55 up-regulated and 6 down-regulated) in N-cells; 40 genes (20 up-regulated, and 20 down-regulated) in NW-cells and 42 genes (9 up-regulated and 33 down-regulated) in DW-cells. There was a significant change in gene expression as compared to the respective controls (Table 1).

Table 1. Summarises the gene expression of N-, NW- and DW-cells. The total RNA from non-irradiated and irradiated N-, NW- and DW-cells were characterised in triplicates, and the gene expression for each gene in the three samples is denoted as gene up-regulation (black) or gene down-regulation (bold).

<table>
<thead>
<tr>
<th>Key (Black-Up-regulation and Red-Down-regulation)</th>
<th>Normal</th>
<th>Normal Wounded</th>
<th>Diabetic Wounded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmembrane Molecules</td>
<td>CD44, CDH1, HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGA8, ITGAM, ITGB2, ITGB3, ITGB4, MMP14, MMP15, MMP16, NCAM1, PECAM1, SELLP, SELP, SPG7, ITGB5</td>
<td>ITGA3, ITGA6, ITG7, ITGA8, ITGA7, ITGAV, ITGB2, ITGB4, MMP15, PECAM1, SELLP, HAS1, ITGA2, ITGAL, ITGB1, ITGB5, SELE1, SELP, SPG7</td>
<td>MMP15, SELLP, SELLP, SGCE, VCAM1, CD44, HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA5, ITGA7, ITGAV, ITGB1, ITGB3, MMP14, SPG7</td>
</tr>
<tr>
<td>Cell-Cell Adhesion</td>
<td>CD44, CDH1, COL14A1, COL6A2, ICAM1, ITGA8, COL11A1</td>
<td>ITGA8, COL14A1, VCAM1</td>
<td>COL11A1, COL14A1, VCAM1, CD44, COL6A2, CTNNBD1</td>
</tr>
<tr>
<td>Cell-Matrix Adhesion</td>
<td>ADAMTS13, ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGM, ITGB2, ITGB3, ITGB4, THBS3, THBS5</td>
<td>ITGA5, ITGA6, ITGA7, ITGA8, ITGAV, ITGB2, ITGB4, ITGA2, ITGAL, ITGB1, SGCE, SPPI</td>
<td>CD44, ITGA1, ITGA2, ITGA3, ITGA5, ITGAV, ITGB1, ITGB3, SPPI, THBS3</td>
</tr>
<tr>
<td>Other Adhesion Molecules</td>
<td>CNTN1, COL5A1, COL6A1, COL7A1, COL8A1, CTNNA1, FN1, KAL1, LAM1, LAM2, LAM3, LAMC1, THBS2, CLEC3B, VTN, VCAN, CTGF</td>
<td>COL12A1, COL5A1, KAL1, LAMMA, CLEC3B, VCAN, CTGF, CTNND2, LAM1, LAM2, LAMC1, TNC</td>
<td>VCAN, COL12A1, COL16A1, COL5A1, COL6A1, COL7A1, CTNNB1, FN1, KAL1, LAM1, LAM2, LAM3, THBS1</td>
</tr>
<tr>
<td>Basement Membrane Constituents</td>
<td>LAM1, LAM2, LAM3, LAMC1</td>
<td>COL7A1, SPARC, LAM1, LAM2, LAMC1</td>
<td>COL7A1, LAM1, LAM3, LAMB3</td>
</tr>
<tr>
<td>Collagens and ECM Structural Constituents</td>
<td>COL5A1, COL6A1, COL7A1, COL8A1, FN1, KAL1, COL11A1, COL1A1</td>
<td>COL12A1, COL1A1, COL5A1, KAL1, COL14A1</td>
<td>COL11A1, COL14A1, COL12A1, COL16A1, COL5A1, COL6A1, COL7A1, COL14A1, FN1, KAL1</td>
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<tr>
<td>ECM Proteases (MMPs responsible for ECM degradation)</td>
<td>ADAMTS1, ADAMTS13, ADAMTS8, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP2, MMP7, MMP8, MMP9, MMP1</td>
<td>MMP15, MMP2, MMP8, TIMP1, MMP1, MMP10, MMP12, MMP3</td>
<td>ADAMTS8, MMP15, ADAMTS1, MMP1, MMP14, MMP2, MMP3, MMP8, SPG7</td>
</tr>
<tr>
<td>ECM Inhibitors</td>
<td>COL7A1, KAL1</td>
<td>KAL1</td>
<td>COL7A1, KAL1, THBS1</td>
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<td>Other ECM Molecules</td>
<td>HAS1, THBS2, CLEC3B, VTN, VCAN, CTGF</td>
<td>CLEC3B, VCAN, CTGF, ECM, SPPI</td>
<td>HAS1, VCN, CTGF</td>
</tr>
</tbody>
</table>

4. Discussion and Conclusion

Photobiostimulation affects various cellular responses both in vivo and in vitro, as well as gene expression [15-17,22,23] This study focused on the gene expression profile of normal, normal wounded and diabetic wounded human skin fibroblast cell models. The explanation behind this could be
the change in the respiratory chain of mitochondria to release energy in the form of adenosine triphosphate (ATP) as well as reactive oxygen species (ROS) leading to the release of transcription factors stimulating gene transcription and eventually ECM formation, cell proliferation, collagen production, growth factor production, as well as the expression of various proteins [24]. Wound healing is impaired in diabetes due to the poor synthesis of growth factors and proteins [11,25,26], as well as cell-ECM interaction [27]. In this study, the main components affected by photobiostimulation in the ECM and adhesion molecules are the cell adhesion molecules (Integrins, Cadherins, Immunoglobulins and Selectins) as well as ECM molecules (Fibronectin, Collagen, Laminin, Matrix Metalloproteinases (MMPs) and their inhibitors). This study showed that 55 genes in N-cells were significantly up-regulated, while only 20 genes in NW-cells and nine genes in DW-cells were up-regulated. Genes in DW-cells were significantly down-regulated possibly as a result of the ECM dysregulation due to hyperglycaemia. In addition, the inflammatory process is delayed enhancing the release of proteases such as MMPs promoting ECM degradation and vital growth factors and receptors responsible for wound healing. The dysregulation may also prevent integrins from binding with fibronectin, and hence impairing migration [28,29]. According to Matsumoto and colleagues [30] a concentration of 1.5% glucose impaired wound healing in mesothelial cells. Their study showed that photobiostimulation in a paracrine or autocrine fashion regulates gene expression in the ECM in isolated skin fibroblasts in vivo. Similarly, Peplow and colleagues [23] reviewed 17 papers on gene expression in human and animal cell cultures and confirmed that photobiostimulation at green, red, and NIR stimulated gene expression, even though further work needs to be done to elucidate its cellular effects. In addition, McDaniel et al [31] exposed human skin fibroblasts to multiple wavelengths (590/870 nm LED array) and concluded that simultaneous exposure can affect cell metabolism as well as gene expression. They suggested that gene expression patterns in fibroblasts could be influenced by adjusting the wavelength intensities in both visible and NIR treatments.

In conclusion, photobiostimulation modulates gene expression of cell adhesion molecules, integrins, ECM proteins, proteases, and inhibitors involved in the ECM at a wavelength of 830 nm. This study confirms that hyperglycaemia is responsible for impaired wound healing in cell cultures in vitro. Furthermore an in depth comprehension of the molecular and biological processes may create an improved therapeutic protocol for diabetic wounds and future studies conducted on gene modulation and their receptors for improve understanding.

Acknowledgement

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References