Response of low intensity laser irradiation on collagen production in diabetic wounded fibroblast cells *in vitro*

S M Ayuk, N N Houreld and H Abrahamse[1]

[1]Laser Research Centre, Faculty of Health Sciences, University of Johannesburg, P.O. Box 17011, Doornfontein, Johannesburg, 2028, South Africa

Tel: +27 11 559-6406 Fax +27 11 559-6884

Email: habrahamse@uj.ac.za

Abstract. *Background*: Collagen Type I (Col-I) is a major component of the extracellular matrix (ECM) and is important in wound healing processes. Several studies have shown that Low Intensity Laser Irradiation (LILI) biostimulates Col-I synthesis both *in vitro* and *in vivo*. Diabetic patients are known to suffer from slow-to-heal wounds and collagen production in these patients is impaired. This study aimed to determine if LILI affects collagen production and related cellular responses in an *in vitro* diabetic wounded fibroblast model. *Method*: This study was performed on isolated human skin fibroblasts. Different cell models namely; normal and diabetic wounded were used. Cells were irradiated with 5 J/cm^2 at a wavelength of 660 nm and incubated for 48 or 72 hours. Non-irradiated cells (0 J/cm^2) were used as controls. Biological responses were assessed. Cellular viability (Trypan blue), morphology (Bright Field Microscopy), proliferation (VisionBlue Quick Cell Proliferation Assay), and Col-I (Enzyme Linked Immunoabsorbent Assay, ELISA) were assessed. *Results:* Diabetic wounded cells irradiated with 5 J/cm^2 at 660 nm showed a significant increase in cell migration, viability, proliferation and Col-I. *Conclusion*: This study shows that LILI stimulates Col-I synthesis in diabetic wound healing *in vitro* at 660 nm.

1. Introduction

Impaired wound healing stands as a major diabetic complication and non-healing ulcers remain a serious problem in public health and clinical practice. It is common to note that out of about 15% of people with diabetic foot ulcers (DFUs), 3% suffer from lower limb amputation [1]. Wound healing comprises of a series of composite biological processes summarized in three phases namely inflammation, proliferation and tissue remodeling. These phases involves cytokines, chemokines, clotting factors, various cells and growth factors [2, 3] that must occur in the correct order, at a given time and at favorable intensities to ensure proper healing [4]. Fibroblasts secrete Col-I and other types of collagen which make up the major protein found in the ECM, provides structural scaffold of the cell that maintains the cell integrity and tensile strength in wound healing [5-7]. Studies have shown that diabetes affects collagen production as well as wound healing [8].

In the visible and Near Infra-Red (NIR) spectral range, Low Intensity Laser Irradiation (LILI) is known to accelerate wound healing processes in cell culture, *in vivo* and animal models [9, 10] at different wavelengths and doses [11]. This process is non-invasive and is achieved at wavelengths between 500-1,100 nm and power outputs of 10-200 mW during treatment [10]. Even though LILI is widely used in medical practice, however, there are disputable views on the use of treatment specifications since there is not enough scientific evidence and guidelines. More so, there is a need for these specifications to be stabilized under optimal conditions in both animal models and clinical practice; hence there is need for more research [12].

[1] To whom all correspondence should be addressed.

The purpose of this study is to determine if LILI affects collagen production and related cellular responses in an *in vitro* diabetic wounded fibroblast model at 660 nm with a fluence of 5 J/cm^2.

2. Methods

2.1 Cell Culture and Laser Irradiation

In this study, normal (N) and diabetic wounded (DW) cells were utilized. Using standardized culture methods [13-16] isolated human skin fibroblast cells (Academic ethics Committee, Clearance Reference Number: 01/06, University of Johannesburg) were regularly cultured. Approximately 6 x 10^5 cells were seeded into 3.3 cm diameter culture plates containing 3 ml complete media and incubated overnight for the cells to attach. Prior to irradiation, a central scratch was created from a confluent cellular monolayer by using a 1 ml sterile pipette to mimic a wounded model [17]. An *in vitro* diabetic wounded model was also established [18]. The normal cells were used as controls. Cells were irradiated using 660 nm diode laser (RGBlase, TECIRL-100G-650SMA, Fremont, California, USA) with a continuous wave emission, 92.8 mW power output, 9.1 cm^2 spot size, 10.22 mW/cm^2, and the cells were exposed to laser irradiation for 8 min 9 s in order to achieve a fluence of 5 J/cm^2. The effects of laser irradiation on cellular responses for cellular morphology, viability, proliferation and collagen production were observed after 48 h and 72 h (See Table 1). All lasers were supplied by the National Laser Center (NLC) of South Africa. Irradiations were performed four times (n=4) and each biochemical assay was done in duplicate and the mean used.

3. Results

Table 1. Summary of the methodology and results

Method	**Data**	**48 h**			**72 h**		
		N 0 J/cm^2	**DW 0 J/ cm^2**	**DW 5 J/cm^2**	**N 0 J/cm^2**	**DW 0 J/cm^2**	**DW 5 J/cm^2**
Trypan blue exclusion test	Viability (%)	95 ± 0.8	94 ± 1.8	97 ± 0.1*	83 ± 0.2	94 ± 0.3***	93 ± 0.1***†
Proliferation MTT (Cell Titre Non-Radioactive Proliferation Assay)	$A_{550\ nm}$	0.163 ± 1.32	0.19 ± 7.5***	0.2 ± 4.8***†††	0.23 ± 3.6	0.31 ± 5.5***	0.26 ± 3.8***†††
VisionBlue (VisionBlue™ Quick Cell Proliferation Assay)	Ex/Em $_{560/595\ nm}$	193 ± 413	225 ± 1513***	237 ± 114***†††	157 ± 0.3	237 ± 222***	213 ± 86***†††

*P<0.05, and ***P<0.001, compared to normal 0 J/cm^2
†P<0.05 and ††† P<0.001, compared to diabetic wounded 0 J/cm^2
± SE

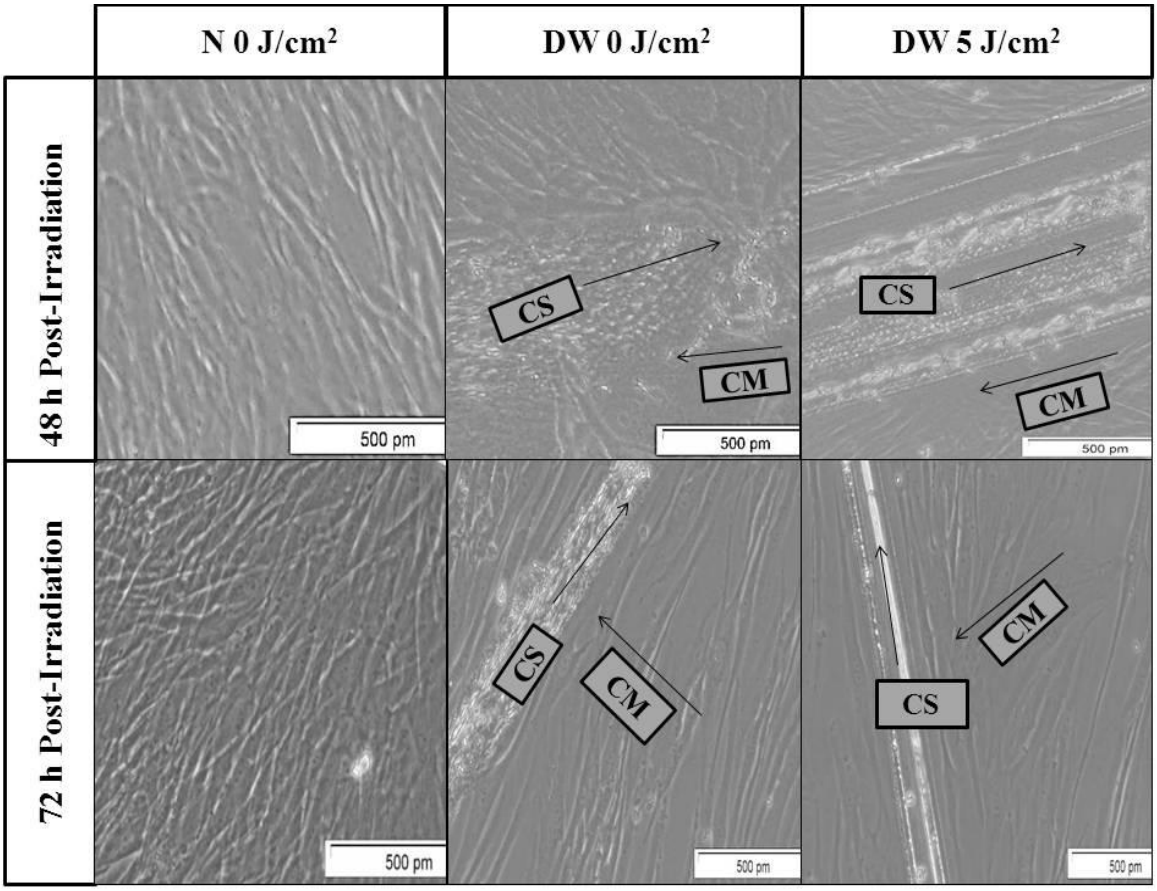

Figure 1. Cell Morphology in an *in vitro* normal, and diabetic wounded models. Fibroblasts appear as slender, spindle shaped cells. The wound, or central scratch (CS) and the direction of cell migration (CM) can clearly be seen.

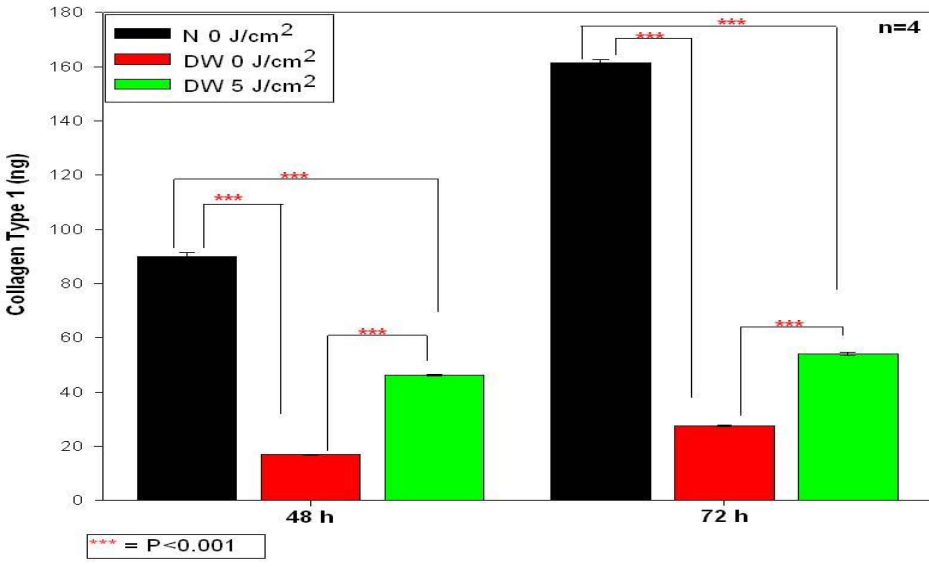

Figure 2. Col I production in an *invitro* normal and diabetic wounded model. Col I was assessed using the Enzyme Linked Immunoabsorbent Assay. Significant probability is shown as *** P<0.001.

4. Discussion and Conclusion

Cell migration, viability, and proliferation are various aspects that affect LILI *in vivo* and *in vitro* [19]. In this study, irradiated diabetic wounded cells showed an increase in cell migration, viability and proliferation compared to non-irradiated diabetic wounded cells, with no changes in morphology (Figure 1). Col-I increased in irradiated diabetic wounded cells compared to non-irradiated cells at 48 h and 72 h (Figure 2). Normal cells also showed a significant increase compared to diabetic wounded cells. According to Karu (2003) [20], LILI is thought to enhance these biological processes. Increase in cell proliferation could possibly have occurred through changes in the mitochondrial respiratory chain as a primary response to healing leading to increased ATP production. This in turn affects RNA synthesis and changes the expression of various proteins [21, 22]. However in a diabetic situation the process is impaired due to disturbed cell migration. As a result of this disorder, there is poor synthesis of growth factors and vasodilator agents [23].

These results showed that LILI increase Col-I production at 660 nm at a fluence of 5 J/cm^2. These findings are in agreement with Silveira and colleagues (2011) [11] who also observed that LILI produced a stimulatory effect in an animal model at 1 and 3 J/cm^2 [24, 25]. Their study further confirmed that shorter wavelengths and energy densities between 1-5 J/cm^2 increase the tensile force of tendons and speed up collagen production compared to higher energy densities and wavelengths [11].

In conclusion, this study adds to evidence that LILI stimulates Col-I synthesis in diabetic wound healing *in vitro* at 660 nm.

5. Acknowledgement

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References

[1] Boulton A J, Vileikyte L, Ragnarson T G and Apelqvist J 2005 *Lancet* 366 1719
[2] Henry G. and Garner W.L. 2003. *Surg Clin North Am.* 83 483
[3] Cross K.J and Mustoe T.A. 2003 Surg Clin North Am. 83 531
[4] Mathieu D, Linke J-C, Wattel F. 2006 pp. 401-427.
[5] Ottani V, Raspanti M and Ruggeri A. 2001 *Micron* 32 251
[6] Rhee S. 2009 *Exp. Mol. Med*. 41 858
[7] Campos A C, Groth A K and Branco A B. 2008 *Curr Opin Clin Nutr Metab Care.* 11 281
[8] Cruz J W, Oliveira M A, Hohman T C and Fortes Z B 2000 *Eur. J. Pharmacol*. 391 163
[9] Huang Y Y, Chen A C H, Carroll J D and Hamblin M R 2009 *Dose Response* 7 358
[10] Hawkins Evans D and Abrahamse H 2009 *Proceedings of BioS (BO109) SPIE-Photonics West (SPIE, Bellingham, WA)* Paper 7165-8
[11] Silveira P C L, Silva L A, Freitas P T, Latini A, Pinho R A 2011 *Lasers Med Sci* 26 125
[12] Silva Junior A N, Pinheiro A L, Oliveira M G, Weismann R, Ramalho L M and Nicolau R A 2002 *J. Clin. Laser Med. Surg*. 20 83
[13] Hawkins D and Abrahamse H 2005 *J Photomed Laser Surg* 23 251
[14] Hawkins D and Abrahamse H 2006 *J Photomed Laser Surg* 24 715
[15] Hawkins D and Abrahamse H 2007 *J Laser Appl* 19 74
[16] Houreld N and Abrahamse H 2010 *Diabetes Technol Ther*. 12 971
[17] Rigau J, Sun C, Trelles M A and Berns M 1996 *Proc SPIE* 2630 38
[18] Houreld N and Abrahamse H 2007 *Photomed Laser Surg* 25 78
[19] Xu X, Zhao X, Liu T C Y and Pan H 2008 *Photomed Laser Surg* 26 197
[20] Karu T 2003 Volume 48. pp 1

[21] Lavi R, Shainberg A, Friedmann H, Shneyvays V, Rickover O, Eichler M, Kaplan D and Lubart R 2003 *J Biol Chem* 278 40917
[22] Gavish L, Asher Y, Becker Y and Kleinman Y 2004 *Lasers Surg Med* 35 369
[23] Kofler S, Nickel T and Weis M 2005 *Clinical sci*. 108 205
[24] Maiya G A, Kumar P and Rao L 2005 *Photomed Laser Surg* 23 187
[25] Carvalho P T, Mazzer N, Reis F A, Belchior A C and Silva I S 2006 *Acta Cir Bras* 21 176