

Effect of Laser Irradiation on Diabetic Wounded Fibroblast Cells in Vitro

N. Houreld and H. Abrahamse

*Laser Research Group, Faculty of Health Sciences, University of Johannesburg,
P.O. Box 17011, Doornfontein, 2028, South Africa Email: nhoureld@uj.ac.za*

Summary

Diabetes is known to be associated with impaired wound healing, and loss of collagen related to diabetes may be due to decreased levels of synthesis or enhanced metabolism of newly synthesized collagen or both. This study aims to determine if laser irradiation stimulates cellular proliferation, nitric oxide (NO) and collagen synthesis in diabetic fibroblast cells. Induced diabetic wounded human fibroblast cells were irradiated at 830 nm with 5 J/cm². Post-laser irradiation there was a significant increase in proliferation at 24 and 48 h and reactive oxygen species and NO generation at 15 min. There was no change in collagen type I. Laser irradiation at 830 nm with 5 J/cm² significantly stimulates cellular proliferation and NO synthesis in diabetic wounded fibroblast cells.

Introduction

Normal wound healing requires both destructive and reparative processes in controlled balance aimed at reversing the loss of structural integrity. During wound healing, collagen synthesis is important during the remodeling phase, where new extracellular matrix (ECM) is synthesized. This fine balance is regulated by matrix metalloproteinases (MMPs), which destroy collagen, and their inhibitors tissue inhibitor metalloproteinase (TIMPs). Collagen synthesis begins on the rough endoplasmic reticulum (RER) with the production of three pro- α -chains, which are then hydroxylated, and glycosylated in the Golgi. Formation of 4-hydroxylproline in these procollagen chains is catalyzed by prolyl-4-hydroxylase (P4H, EC 1.14.11.2). Procollagen is formed from the α -chains that fold into a triple-helical conformation, is secreted from vesicles, and undergoes proteolysis at its ends in the extracellular space. Collagen

molecules are then cross linked into fibrils which then self-assemble into fibres.¹

Nitric oxide (NO) has been considered to have a biphasic effect in pathological conditions being both beneficial and detrimental depending on the concentration. NO has been shown to down-regulate ECM proteins, such as type I collagen,^{2,3} at the same time, in early wound healing, NO favours collagen synthesis and the formation of granulation tissue. Fibroblasts isolated from healing wounds synthesize NO spontaneously and inhibition of NO synthesis decreases collagen synthesis.⁴⁻⁶ Diabetes is known to be associated with impaired wound healing, and is associated with a variety of alterations in connective tissue metabolism. Loss of collagen related to diabetes may be due to decreased levels of synthesis or enhanced metabolism of newly synthesized collagen or both.⁷ NO is significantly reduced in chronic ulcers and impaired healing of diabetic wounds is thought to be related to this decrease.^{8,9} Burrow *et al.*, demonstrated that normal skin fibroblasts produce more NO than diabetic human skin fibroblasts, and that there was a direct relationship between NO levels and MMP expression.¹⁰

Various studies show that phototherapy modulates collagen and NO synthesis both *in vitro* and *in vivo*. Gavish *et al.*, found an increase in collagen synthesis in porcine aortic smooth muscle cells at 780 nm.¹¹ Maiya *et al.*, demonstrated an increase in collagen in diabetic rats (632.8 nm),¹² which corresponded with the work of Carvalho and colleagues.¹³ Zhu *et al.*, and Chi *et al.*, showed direct evidence of NO generation in illuminated cells.^{14,15} Since NO has been linked to ECM synthesis, it would appear plausible that laser phototherapy may influence collagen synthesis via NO.

Materials and Methods

Human skin fibroblast cells (WS1, ATCC CRL1502) were grown according to standard culture techniques. A diabetic model was achieved by growing cells in minimal essential media (basal glucose of 5.6 mMol/L) containing an additional 17 mMol/L glucose.¹⁶⁻¹⁸ A wound was simulated whereby the monolayer of cells was scratched using a sterile pipette.^{17,19} Approximately 6×10^5 cells in 3.3 cm diameter culture plates were irradiated in the dark using a 830 nm diode laser with a dose of 5 J/cm² (spot size 9.08 cm²). Unirradiated normal wounded and diabetic wounded cells were used as controls. The study design is summarized in Table 1. Cellular proliferation was examined using

Table 1 Study design (n=4).

	Proliferation	Collagen I	NO ^a	ROS ^b
Incubation Time	24 or 48 h	24 or 48 h	15 min or 1 h	15 min
Method	Fluorescence	ELISA	Griess reagent system	Immunofluorescent staining
Data Collection	Fluorescent spectroscopy	Spectroscopy	Spectroscopy	Fluorescence microscopy

NO^a Nitric oxide ROS^b Reactive oxygen species

the VisionBlue™ Fluorescent Cell Viability Assay (BioVision, K303-500), NO by the Griess Reagent System (Promega, G2930), collagen type I by ELISA and reactive oxygen species (ROS) using the Image-iT Live Green Reactive Oxygen Species Detection Kit (Invitrogen, I36007).

Results

Irradiated diabetic wounded cells showed a significant increase in proliferation at 24 ($p < 0.001$) and 48 h ($p < 0.01$) as compared to both normal wounded and diabetic wounded unirradiated control cells (Fig 1). ELISA did not reveal any significant changes in collagen type I at 24 or 48 h (Fig 2). Cells incubated for 48 h showed an increase in both proliferation and collagen compared to cells incubated for 24 h ($p < 0.001$ and $p < 0.01$ respectively). Staining of WS1 cells for ROS revealed an increase in staining in unirradiated and irradiated diabetic cells (Fig 3). Diabetic cells irradiated with 5 J/cm^2 showed positive ROS staining comparable to the positive control ($100 \mu\text{M}$ *tert*-butyl hydroperoxide). Diabetic wounded cells incubated at 37°C for 15 min post-laser irradiation showed a significant increase in NO compared to both normal wounded and diabetic wounded unirradiated cells ($p < 0.01$), (Fig 4). There was no significant change when cells were incubated for 1 h. There was a significant decrease in NO ($p < 0.01$) in irradiated diabetic wounded cells incubated for 1 hour compared to cells incubated for 15 min.

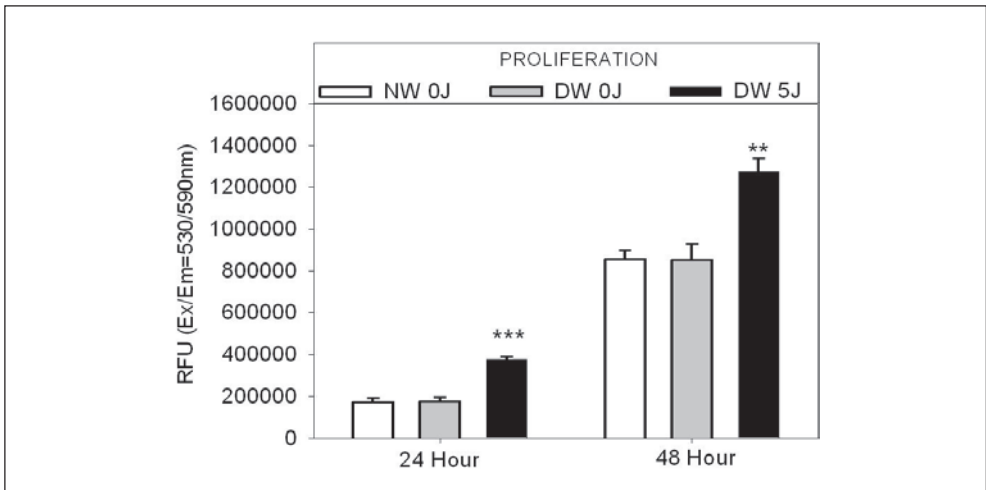


Fig 1. Cellular proliferation was determined in diabetic wounded human skin fibroblast cells 24 and 48 h post-laser irradiation (DW 5J). Normal wounded (NW 0J) and diabetic wounded (DW 0J) unirradiated cells served as controls. There was a significant increase in relative fluorescent units (RFU), and hence proliferation, in irradiated cells compared to control cells. Proliferation was increased at 48 h in all cell types ($p < 0.001$).

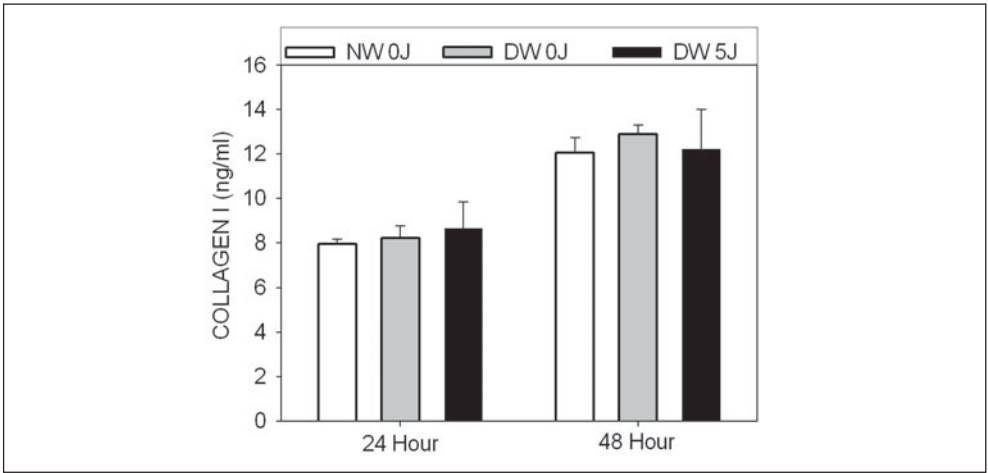


Fig 2. Collagen type I was determined by ELISA in diabetic wounded human skin fibroblast cells 24 and 48 h post-laser irradiation (DW 5J). Normal wounded (NW 0J) and diabetic wounded (DW 0J) unirradiated cells served as controls. Laser irradiation had no effect on collagen synthesis at 24 and 48 h.

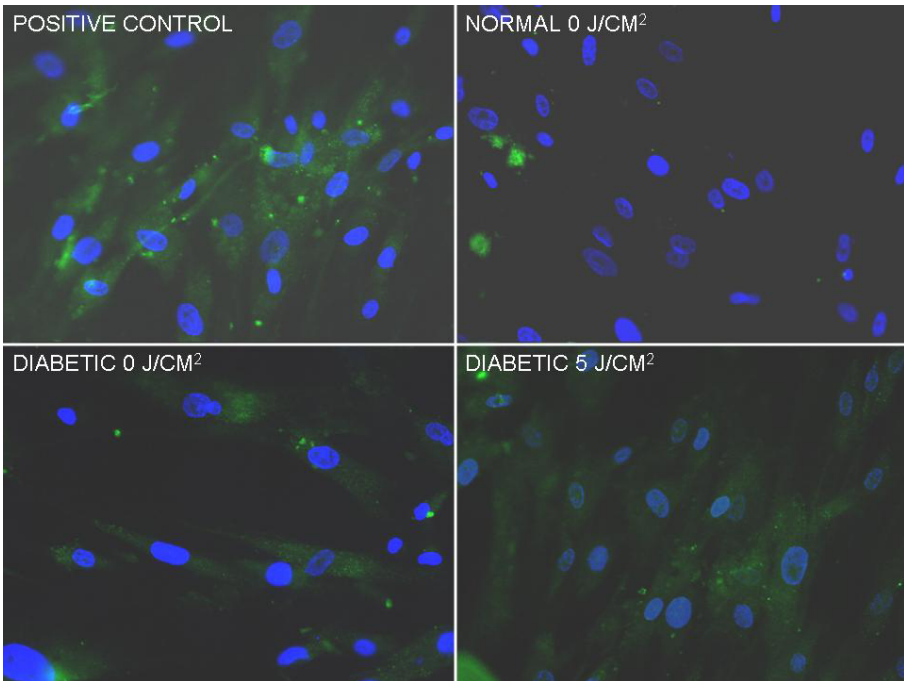


Fig 3. The generation of reactive oxygen species (ROS) was determined by immunofluorescent staining in diabetic fibroblast cells 15 min post-laser irradiation. Normal and diabetic unirradiated cells served as controls, and treatment with 100 μ M tert-butyl hydroperoxide served as a positive control. Cells irradiated with 5 J/cm² showed an increase in ROS generation. Diabetic unirradiated cells showed an increase in ROS compared to normal unirradiated cells, however the generation of ROS was not as much as in irradiated cells.

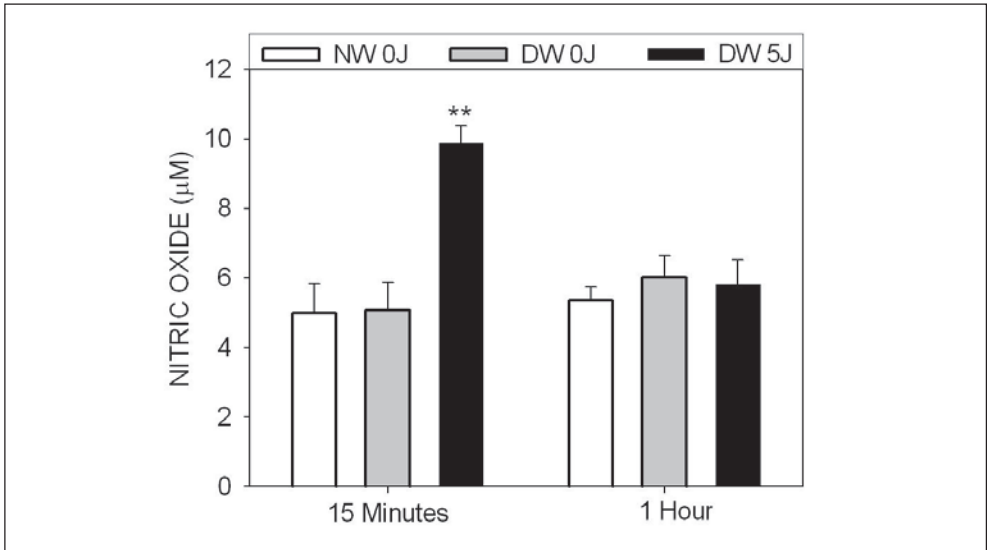


Fig 4. Nitric oxide (NO) was determined in diabetic wounded human skin fibroblast cells 15 min and 1 h post-laser irradiation (DW 5J). Normal wounded (NW 0J) and diabetic wounded (DW 0J) unirradiated cells served as controls. There was a significant increase at 15 min, while at 1 h there was no significant change. There was a significant decrease in irradiated cells at 1 h compared to 15 min ($p < 0.01$).

Conclusion

In vitro irradiation of diabetic wounded fibroblast cells at a wavelength of 830 nm with 5 J/cm^2 stimulates wound healing. There was an increase in migration (results not shown), proliferation and NO generation. There was a significant increase in NO 15 min post-irradiation and no change at 1 h, suggesting NO is released directly by a photochemical mechanism.²⁰ Changes in the cellular redox state can modulate many biological processes, including proliferation. There was no significant changes between non-irradiated normal wounded and diabetic wounded cells, except in ROS staining. Despite literature showing an increase in collagen post-laser irradiation, this study could not show any differences at 24 or 48 h. However there was an increase at 48 h compared to 24 h. A longer incubation time (e.g. 72 h) or a change in irradiation parameters might produce a change in these results since there is strong evidence in the literature that laser irradiation stimulates collagen synthesis in a variety of cell types. This paper cannot link laser irradiation, NO generation and collagen synthesis, since there is an increase in ROS and NO, but no significant increase in collagen type I. Laser irradiation of diabetic wounded fibroblast cells has a positive effect on wound healing, cellular proliferation and ROS generation, including NO.

References

1. Li H., Huang C., Chen S. and Chou M. Assembly of homotrimeric type XXI minicollagen by coexpression of prolyl 4-hydroxylase in stably transfected *Drosophila melanogaster* S2 cells. *Biochemical and Biophysical Research Communications* 336: 375–385, 2005.
2. Chu A.J. and Prasad J.K. Up-regulation by human recombinant transforming growth factor α -1 of collagen production in cultured dermal fibroblasts is mediated by the inhibition of nitric oxide signalling. *Journal of the American College of Surgeons* 188(3): 271-280, 1999.
3. Kim N.N., Villegas S., Summerour S.R. and Villarreal F.J. Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. *Journal of Molecular and Cellular Cardiology* 31(2): 457–466, 1999.
4. Shi H.P., Efron D.T., Most D. and Barbul The role of iNOS in wound healing. *Surgery* 130(2): 225-229, 2001.
5. Stallmeyer B., Anhold M., Wetzler C., et al. Regulation of eNOS in normal and diabetes-impaired skin repair: Implications for tissue regeneration. *Nitric Oxide* 6(2): 168-177, 2002.
6. Kapoor M., Kojima F., Appleton I., Kawai S. and Crofford L.J. Major enzymatic pathways in dermal wound healing: Current understanding and future therapeutic targets. *Current Opinion in Investigational Drugs* 7(5): 418-422, 2006.
7. Arul V., Kartha R. and Jayakumar R. A therapeutic approach for diabetic wound healing using biotinylated GHK incorporated collagen matrices. *Life Sciences* 80: 275–284, 2007.
8. Bulgrin J.P., Shanbani M., Chakravarthy D. and Smith D.J. Nitric oxide synthesis is suppressed in steroid-impaired and diabetic wounds. *Wounds* 7: 48, 1995.
9. Schwentker A., Vodovotz Y., Weller R. and Billiar T.R. Nitric oxide and wound repair: role of cytokines? *Nitric Oxide* 7: 1-10, 2002.
10. Burrow J.W., Koch J.A., Chuang H., et al. *Journal of Surgical Research* 140(1): 90-98, 2007.
11. Gavish L., Perez L. and Gertz S.D. Low-level laser irradiation modulates matrix metalloproteinase activity and gene expression in porcine aortic smooth muscle cells. *Lasers in Surgery and Medicine* 38: 779-786, 2006
12. Maiya G.A., Kumar P. and Rao L. Effect of low intensity helium-neon (He-Ne) laser irradiation on diabetic wound healing dynamics. *Photomedicine and Laser Surgery* 23(2): 187-190, 2005.
13. Carvalho P.T., Mazzer N. dos Reis F.A., Belchior A.C. and Silva I.S. Analysis of the influence of low-power HeNe laser on the healing of skin wounds in diabetic and non-diabetic rats. *Acta Cir Bras.* 21(3):177-83, 2006.
14. Zhu, Q., Yu Wei, X., Hicks, G.L., et al. Photo-irradiation improved functional preservation of isolated rat heart. *Lasers Surg. Med.* 20: 332–339, 1997.
15. Chi, L.H., Yu, W., Naim, J.O., et al. Increase synthesis of nitric oxide by laser irradiation in sepsis. *Lasers Surg. Med. Suppl* 7, 19, 1995.
16. McDermott A.M., Kern T.S. and Murphy C.J. The effect of elevated extracellular glucose on migration, adhesion and proliferation of SV40 transformed human corneal epithelial cells. *Curr. Eye Res* 17(9): 924 – 932, 1998.
17. Hamuro M., Polan J., Natarajan M. and Mohan S. High glucose induced nuclear factor kappa B mediated inhibition of endothelial cell migration. *Atherosclerosis* 162(2): 277 – 287, 2002.
18. Vinck E.M., Cagnie B.J., Cornelissen M.J., Declercq H.A. and Cambier D.C. Green light emitting diode irradiation enhances fibroblast growth impaired by

high glucose level. *Photomed Laser Surg* 23(2): 167 – 171, 2005

19. Rigau J., Sun C., Trelles M.A. and Berns M. Effects of the 633nm laser on the behavior and morphology of primary fibroblasts in culture, Karu T. and Young A. (Eds) *Effects of low power light on biological systems*. Barcelona Spain, Progress in Biomedical Optics, pp 38 – 42, 1995.
20. Lubart R., Eichler M., Lavi R., Friedman H., And Shainberg A., Low-Energy Laser Irradiation Promotes Cellular Redox Activity. *Photomed Laser Surg* 23(1): 3-9, 2005.