Laser irradiation in the visible wavelength stimulates wound healing in vitro

Houreld N.N.

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

Summary

Low intensity laser irradiation (LILI) has been applied to stimulate healing in a variety of conditions, such as diabetes. This study aimed to determine the biochemical and molecular responses to LILI in the visible red spectrum. Various cell culture models, namely wounded, diabetic wounded and hypoxic/ischemic were used. Models were exposed to visible red laser light (632.8, 636 or 660 nm) at a fluence of 5 J/cm^2. Post-irradiation, the effect on cells was studied. Laser irradiation was shown to have a positive effect on stressed cells in vitro. There was an increase in migration, cell survival and proliferation, mitochondrial activity, secondary messengers and collagen. A decrease in cytotoxicity, DNA damage and pro-inflammatory cytokines were also seen. LLLT offers an alternative wound healing therapy. At a biochemical level there was a positive effect on cells, with stressed cells being pushed into cell survival pathways.

Introduction

Since the invention of the laser, its application in the health sector has been studied in an attempt to discover effective alternative treatments. Low intensity laser irradiation (LILI) has been applied to stimulate healing in a variety of conditions, such as diabetes. Although this therapy is in use worldwide, the full cellular and molecular mechanisms of action are not fully understood.

Mitochondria are involved in cellular respiration and the formation of adenosine triphosphate (ATP) via the electron transport chain (ETC). Mitochondria are also involved in programmed cell death. Light in the visible red spectrum (620-750 nm) is thought to be absorbed by mitochondria. When photon energy is absorbed by mitochondria, a cascade of events is set into action, including ATP synthesis, changes in membrane permeability and mitochondrial membrane potential (MMP), and release of nitric oxide (NO) and intracellular calcium (Ca^{2+})i.[1,2] It has been postulated that the photon energy is absorbed by the mitochondrial enzyme, cytochrome c oxidase (complex IV), which begins vibrating and alters the redox reactions resulting in an increase in electron transfer which in turn results in increased oxidative metabolism and (Ca^{2+})i.[1,3] Karu *et al.*,[3] showed that irradiation at 632.8 nm causes the reduction or oxidation of cytochrome c oxidase and this is dependent on the initial redox state of the enzyme at the time of irradiation. A number of studies have shown that LILI affects mitochondrial metabolism by measuring MMP,[4] oxygen consumption,[5] ATP[6] and mitochondrial enzyme activity.[7,8]

Secondary messengers are short-lived intracellular molecules which relay signals to intracellular targets often leading to rapid alterations in cellular enzyme activity.[9] Adenosine 3',5'-cyclic monophosphate (cAMP) and (Ca^{2+})i are examples of such messengers. cAMP is generated from ATP and is involved in the phosphorylation of cellular proteins. (Ca^{2+})i regulates many processes and has a direct effect on cellular motility. Laser light is absorbed by photoacceptors which leads to changes in membrane permeability which in turn trigger the release of secondary messengers which initiate a wide variety of biological processes.[9]

Materials and methods

Human skin fibroblast cells (WS1, ATCC CRL1502) were grown according to standard culture techniques. For experiments, 6x10^5 cells were seeded into 3.4 cm diameter culture plates. A wound model was achieved through the central scratch method, whereby a confluent monolayer of cells was scrapped with a sterile 1 ml pipette.[10] A diabetic model was achieved by continuously growing cells in minimal essential media (MEM) containing an additional 17 mMol/L glucose.[11] A state of hypoxia/ischemia was induced *in vitro* by culturing cells for 24 h in serum free media followed by a 4 h incubation in an anaerobic atmosphere.[2,12]

Cells were irradiated from above, with the culture dish lids off, and in the dark using a 632.8, 636 or 660 nm laser with a fluence of 5 J/cm^2 (Table 1). Unirradiated cells were sham-irradiated and used as controls. Cells

were incubated for variable periods post-irradiation, depending on the parameter being measured. The study design is summarized in Table 2.

Results

Irradiation of wounded and diabetic wounded cells at wavelengths in the visible red spectrum resulted in hastened cell migration and wound closure. Hypoxic/ischemic cells showed considerable cellular damage, with cells appearing irregular in shape, rounding off and detaching from the tissue culture plate. Post-irradiation, these cells began to regain their normal cellular morphology, with fewer cells detaching and less spaces seen between cells.

Table 1 Laser parameters.

Wavelength (nm)	632.8	636	660
Light source	Helium-Neon	Diode laser	Diode laser
Wave emission	Continuous wave	Continuous wave	Continuous wave
Spot Size (cm^2)	9.1	9.1	9.1
Power output (mW)	20	95	100
Power density (mW/cm^2)	2.2	10.44	11
Irradiation time (s)	2,273	479	455
Energy density (J/cm^2)	5	5	5

Table 2 Study design (n=4).

Parameter	**Assay**	**Data Collection**
Morphology	Cellular migration	Light microscopy
Apoptosis	Caspase 3/7	Luminescence
Viability	Trypan blue staining	Light microscopy
	ATP	Luminescence
Proliferation	XTT	Colorimetric (A_{450} nm)
	MTT	Colorimetric (A_{550} nm)
	VisionBlue	Ex/Em 560/595 nm
	Basic fibroblast growth factor (bFGF)	ELISA (A_{450} nm)
	Alkaline phosphatase (ALP)	Colorimetric (A_{405} nm)
Cytokines	Interleukin (IL)-6	ELISA (A_{450} nm)
		Flow Cytometry
	Interleukin (IL)-1-beta (β)	ELISA (A_{450} nm)
		Flow Cytometry
	Tumour Necrosis Factor-alpha (α)	ELISA (A_{450} nm)
		Flow Cytometry
Mitochondria	Complex I-V activity	Enzyme kinetics
	Complex I-IV transcription	RT-PCR
	Membrane potential	Flow Cytometry
Secondary messengers	cAMP	ELISA (A_{405} nm)
	Colorimetric (A_{405} nm)	
DNA damage	Comet assay	Fluorescent microscopy
Collagen	Collagen type I	ELISA (A_{450} nm)
Cytotoxicity	Lactate dehydrogenase (LDH)	Colorimetric (A_{490} nm)

When normal, wounded, diabetic wounded or hypoxic cells were irradiated at 636 nm with a fluence of 5 J/cm^2 there is an increase in cellular viability and proliferation, and a decrease in caspase 3/7 activity, as well as the inflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-1β. Hypoxic cells

showed a significant increase in IL-6.[12] Wounded cells showed no significant change in DNA damage.[13]
Irradiation of normal, diabetic and ischemic mitochondria at 660 nm with 5 J/cm^2 produced no significant changes in the enzyme activities of complex I, II or III. There was a significant increase in complex IV in normal and diabetic mitochondria.[2] Irradiated normal cells showed a significant increase in ATP.[2] Quantitative real-time reverse transcriptase (RT) polymerase chain reaction (PCR) showed that COX6C, ATP5F1, NDUFA11 and NDUFS7 were significantly up-regulated in wounded fibroblast cells. COX6B2, COX6C and PPA1 were significantly up-regulated in diabetic wounded cells, while ATP4B and ATP5G2 were up-regulated in ischemic cells.[14] When left to incubate for 48 h post laser irradiation, diabetic wounded cells showed an increase in viability and proliferation. The same cells showed a significant increase in collagen type I when left to incubate for 48 or 72 h.[15]
Exposure of cells to irradiation at 632.8 nm resulted in increased MMP in wounded and hypoxic cells. Wounded cells showed an increase in ATP and cAMP while wounded and hypoxic cells showed an increase in (Ca^{2+})i. There was no significant change in lactate dehydrogenase (LDH), and hence cytotoxicity, in wounded, hypoxic and diabetic wounded cells.[1] Diabetic wounded cells showed a significant increase in cellular proliferation (bFGF, ALP, IL-6),[11,16] and no change in LDH and DNA damage.[17]

Conclusion

Irradiation of human skin fibroblasts with visible red laser light positively affects stressed cells. There was increased migration of cells and complete wound closure in irradiated wounded models, and normalisation of cellular features in stressed, ischemic/hypoxic models. This may be as a result of the increase in (Ca^{2+})i.

Mitochondria are known as the powerhouse of the cell and are involved in a number of metabolic pathways, such as apoptosis, cellular differentiation, division, growth and signaling, and more importantly the production of ATP through the ETC. There is a definite influence on cellular mitochondria. LILI increases complex IV (cytochrome c oxidase) enzyme activity resulting in increased ATP. Irradiation not only affects mitochondrial enzymes directly, but also stimulates the up-regulation of genes involved in the ETC and oxidative phosphorylation. NDUFA11 and NDUFS7 are involved in complex I (NADH; ubiquinone oxidoreductase), COX6B2 and COX6C in complex IV (cytochrome c oxidase), PPA1 and ATP5F1 in oxidative phosphorylation, and ATP4B and ATP5G2 both encode subunits of ATP synthase. There is an increase in MMP, (Ca^{2+})i, ATP and cAMP.
Irradiation of stressed fibroblast cells to visible red laser light at a fluence of 5 J/cm^2 does not produce any further cellular and nuclear damage, nor induce apoptosis, as seen by the non-significant changes in LDH, DNA damage and caspase 3/7 respectively. There is a decrease in inflammatory cytokines (TNF-α and IL-1β) and an increase in cellular viability and proliferation.

Collagen is a major structural protein in the extracellular matrix (ECM) and is essential in wound healing. There is a decrease in collagen in diabetic wound healing, and this is due to decreased production and or increased destruction. Irradiation of diabetic wounded cells to 660 nm stimulates collagen type I production.
LILI at a fluence of 5 J/cm^2 and a wavelength in the visible red spectrum positively effects wound healing in stressed models, normalises cellular function, and directs cells into cell survival pathways.

References

1. Zungu I.L., Hawkins Evans D., Abrahamse H. Mitochondrial responses of normal and injured human skin fibroblasts following low level laser irradiation – an in vitro study. *Photochem Photobiol* 85:987-996, 2009.
2. Houreld N.N., Masha R.T. and Abrahamse H. Low-intensity laser irradiation at 660 nm stimulates cytochrome c oxidase in stressed fibroblast cells. *Lasers Surg Med* 44:429–434, 2012.
3. Karu T.I., Pyatibra L.V., Kolyakov S.F. and Afanasyeva N.I. Absorption measurements of cell monolayers relevant to meachanisms of laser phototherapy: Reduction or oxidation of cytochrome c oxidase under laser radiation at 632.8 nm. *Photomed Laser Surg* 26:593-599, 2008.
4. Gavish L, Asher Y, Becker Y, Kleinman Y. Low level laser irradiation stimulates mitochondrial membrane potential and disperses subnuclear promyelocytic leukemia protein. *Lasers Surg Med* 35:369–376, 2004.
5. Hagen TM, Yowe DL, Bartholomew JC, Wehr CM, Do KL. Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase. *Proc Natl Acad Sci USA* 94:3064–3069, 1997.
6. Hu WP, Wang JJ, Yu CL, Lan CCE, Chen GS, Yu HS. Helium-Neon laser irradiation stimulates cell proliferation through photostimulatory effects in mitochondria. *J Invest Dermatol* 127:2048–2057, 2007.

7. Silveira PCL, Streck EL, Pinho RA. Evaluation of mitochondrial respiratory chain activity in wound healing by low-level laser therapy. *J Photochem Photobiol B: Biology* 86:279–282, 2007.
8. Yu W, Naim JO, McGowan M, Ippolito K, Lanzafame RJ. Photomodulation of oxidative metabolism and electron chain enzymes in rat liver mitochondria. *Photochem Photobiol* 66:866–871, 1997.
9. Hawkins Evans D. and Abrahamse H. A review of laboratory-based methods to investigate second messengers in low-level laser therapy (LLLT). *Medical Laser Application* 24:201-215, 2009.
10. 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.
11. Houreld N. and Abrahamse H. Irradiation with a 632.8 nm helium-neon laser with 5 J/cm^2 stimulates proliferation and expression of Interleukin-6 in diabetic wounded fibroblast cells. *Diabetes Technol Ther* 9:451-459, 2007.
12. Sekhejane P.R., Houreld N. and Abrahamse H. Irradiation at 636 nm positively affects diabetic wounded and hypoxic cells in vitro. *Photomed Laser Surg* 29:521-530, 2011.
13. Mbene A.B., Houreld N.N. and Abrahamse H. DNA damage after phototherapy in wounded fibroblast cells irradiated with 16 J/cm2. *J Photochem Photobiol B: Biology* 94:131-137, 2009.
14. Masha R.T., Houreld N.N. and Abrahamse H. Low-intensity laser irradiation at 660 nm stimulates transcription of genes involved in the electron transport chain. *Photomed Laser Surg* Submitted, 2012.
15. Ayuk S.M., Houreld N.N. and Abrahamse H. Collagen production in diabetic wounded fibroblasts in response to low intensity laser irradiation (LILI) at 660 nm. *Diabetes Tech Ther* In Press, 2012.
16. Houreld N. and Abrahamse H. Low-intensity laser irradiation stimulates wound healing in diabetic wounded fibroblast cells (WS1). *Diabetes Tech Ther* 12:971-978, 2010.
17. Houreld N. and Abrahamse H. In Vitro exposure of wounded diabetic fibroblast cells to a Helium-neon laser at 5 and 16 J/cm^2. *Photomed Laser Surg* 25:78-84, 2007.