



Laser photobiomodulation in pressure ulcer healing of human diabetic patients: gene expression analysis of inflammatory biochemical markers

Anelice Calixto Ruh, Lucio Frigo, Marcos Fernando Xisto Braga Cavalcanti, Paulo Svidnicki, Viviane Nogaroto Vicari, Rodrigo Alvaro Brandao Lopes-Martins, Ernesto Cesar Pinto Leal Junior, Natalia de Isla, Francesca Diomede, Oriana Trubiani, et al.

► To cite this version:

Anelice Calixto Ruh, Lucio Frigo, Marcos Fernando Xisto Braga Cavalcanti, Paulo Svidnicki, Viviane Nogaroto Vicari, et al.. Laser photobiomodulation in pressure ulcer healing of human diabetic patients: gene expression analysis of inflammatory biochemical markers. *Lasers in Medical Science*, 2018, 33 (1), pp.165 - 171. 10.1007/s10103-017-2384-6 . hal-01831939

HAL Id: hal-01831939

<https://hal.univ-lorraine.fr/hal-01831939>

Submitted on 4 Apr 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Lasers in Medical Science

Laser-photobiomodulation in human pressure ulcer healing: gene-expression analysis of inflammatory biochemical markers.

--Manuscript Draft--

Manuscript Number:	
Full Title:	Laser-photobiomodulation in human pressure ulcer healing: gene-expression analysis of inflammatory biochemical markers.
Article Type:	Original Article
Keywords:	LLLT, wound healing, growth factors, pressure ulcer, human
Corresponding Author:	Marcos Fernando Xisto Braga Cavalcanti, PhD Cruzeiro do Sul University Sao Paulo, Sao Paulo BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Cruzeiro do Sul University
Corresponding Author's Secondary Institution:	
First Author:	Anelice Calixto Ruh, PhD
First Author Secondary Information:	
Order of Authors:	Anelice Calixto Ruh, PhD Lúcio Frigo, PhD Marcos Fernando Xisto Braga Cavalcanti, PhD Paulo Svidnicki, PhD Viviane Nogaroto Vicari, PhD Rodrigo Alvaro Brandão Lopes-Martins, PhD Ernesto Cesar Pinto Leal Junior, PhD Natalia De Isla, PhD Francesca Diomede, PhD Oriana Trubiani, PhD Giovani Marino Favero, PhD
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	<p>Background: Pressure ulcers (PU) are wounds located mainly on bone surfaces where the tissue under pressure suffers ischemia leading to cellular lesion and necrosis, its causes and the healing process depend on several factors.</p> <p>Purpose: The aim of this study was to evaluate the gene expression of inflammatory/reparative factors: IL6, TNF, VEGF, and TGF, which take part in the tissue healing process under effects of low-level laser therapy (LLLT).</p> <p>Methods: In order to perform lesion area analysis, PUs were photographed and computer analyzed. Biochemical analysis was performed sampling ulcer border tissue obtained through biopsy before and after laser therapy and Quantitative Real Time PCR (qRT-PCR) analysis. The study comprised eight individuals, mean age sixty-two years old, sacroiliac and calcaneous PU, classified as degree III and IV according to National Pressure Ulcer Advisory Panel (NPUAP). PUs were irradiated with low-level laser (InGaAIP, 100mW, 660nm), energy density 2J/cm², once a day, with intervals of 24 hours, totaling 12 applications.</p> <p>Results: The lesion area analysis revealed averaged improvement of the granulation</p>

	tissue size up to 50% from pre to post-treatment. qRT-PCR analysis revealed IL6 values were not significantly different before and after treatment, TNF gene-expression was reduced and VEGF and TGF- β gene-expression increased after treatment. Conclusion: After LLLT, wounds presented improvement in gross appearance, with increase in factors VEGF and TGF- β , which take part in angiogenesis, attract fibroblasts and stimulate collagen production.
--	---

Abstract

Background: Pressure ulcers (PU) are wounds located mainly on bone surfaces where the tissue under pressure suffers ischemia leading to cellular lesion and necrosis, its causes and the healing process depend on several factors.

Purpose: The aim of this study was evaluating the gene expression of inflammatory/reparative factors: IL6, TNF, VEGF, and TGF, which take part in the tissue healing process under effects of low-level laser therapy (LLLT).

Methods: In order to perform lesion area analysis, PUs were photographed and computer analyzed. Biochemical analysis was performed sampling ulcer border tissue obtained through biopsy before and after laser therapy and Quantitative Real Time PCR (qRT-PCR) analysis. The study comprised eight individuals, mean age sixty-two years old, sacroiliac and calcaneous PU, classified as degree III and IV according to National Pressure Ulcer Advisory Panel (NPUAP). PUs were irradiated with low-level laser (InGaAIP, 100mW, 660nm), energy density 2J/cm², once a day, with intervals of 24 hours, totaling 12 applications.

Results: The lesion area analysis revealed averaged improvement of the granulation tissue size up to 50% from pre to post-treatment. qRT-PCR analysis revealed IL6 values were not significantly different before and after treatment, TNF gene-expression was reduced and VEGF and TGF- β gene-expression increased after treatment.

Conclusion: After LLLT, wounds presented improvement in gross appearance, with increase in factors VEGF and TGF- β , which take part in angiogenesis, attract fibroblasts and stimulate collagen production.

Key-words: LLLT, wound healing, growth factors, pressure ulcer, human.

Introduction

Wound healing is a complex, interactive and integrative event, involving cellular and chemotactic activity with release of chemical mediators associated with vascular responses and cell proliferation. It consists in a sequence of events leading to the closure of dermal injuries. The repair follows steps of inflammation, re-epithelization, wound contraction, and matrix remodeling. The healing process can be influenced by various local and systemic factors ^[1, 2].

Wounds can be classified according to time of tissue repair as acute or chronic. Acute wounds originate from surgery or trauma and repair occurs in a timely manner, without complications. Chronic wounds are those not repaired in expected time and exhibit complications ^[2].

Pressure ulcers (PU) are defined as skin or soft tissue injuries, superficial or deep, ischemic, secondary to increased external pressure, usually present in bone crest. Bone crests are covered by a thin layer of muscle or subcutaneous tissue making it more susceptible to developing PU. The most affected regions are scapula, sacrum, ischium, trochanters and calcaneus ^[1,2].

Many factors interfere in wound healing such as: inadequate nutrition, necrotic tissue, foreign bodies, bacteria, blood supply interference, blockage of the lymph vessels, systemic diseases, diabetes mellitus and psychological factors^[1,3].

Diabetic ulcers are a good example of how physiological and biochemical changes can lead to chronic ulcers. They often occur in patients who are unable to sense and relieve cutaneous pressure due to neuropathy. Secondary ischemia from vascular disease decreases healing, reducing oxygen supply and tissue nutrition. Diabetic patients are also susceptible to infection due impaired function and granulocyte chemotactic ^[3]. Other abnormalities associated with diabetic ulcers include prolonged inflammation, impaired neovascularization, decreased synthesis of collagen, increased levels of proteinases, and defective macrophage function ^[3]. Currently an increasing body of evidence link cytokines and growth factors to wound healing process. Mitogenic factors, like TGF- β are present in wound fluid, but levels do not increase during healing process. On the other hand, cytokines, like IL-6 and TNF- α decrease during healing process. ^[4, 5]

The potential of low-level laser therapy (LLLT) in promoting cell proliferation was firstly reported around 27 years ago, since then several studies (including animal experiments and clinical trials) aiming to investigate LLLT effects in wound healing have been published over the years. ^[6-11]

The effects of laser radiation over living tissues are based on the absorption of its energy and its transduction into a biological process, mainly due ATP synthesis ^[12]. In addition, LLLT may modulate transcription of several growth factors, including FGF and VEGF. ^[13, 14]

In this perspective, the aim of this study was evaluating the effects of LLLT on macroscopic aspect and mRNA expression of genes related to wound healing in diabetic patients presenting pressure ulcers.

Materials and Methods

Research Ethics Committee of Ponta Grossa University (COEP-UEPG) approved this study (protocol number 07897/11, opinion 48/2011), that was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.”

Eight volunteers, patients at Santa Casa de Misericordia Hospital at Ponta Grossa (SCMPG), met the following inclusion criteria: age between 30 to 75 years old, diabetic, pressure ulcer (PU) presenting grade II, III or IV, NPUAP ^[2].

According to NPUAP 2009 ^[2], PS grade III shows significant skin loss involving damage or necrosis of subcutaneous tissue that may extend to the fascia. In grade IV PS a significant loss occurs with extensive destruction of skin and necrosis of subcutaneous tissue and/or muscle, bone or supporting structures like tendons and joint capsules.

Sample

Seven male, one female, average age was 62.5 years, one calcaneus PU grade III and seven PU sacrococcygeal grade III or IV. All subjects in the study were hospitalized in wards or intensive care unit, immobilized in bed, due to primary diseases.

All were volunteers and had different kinds of diseases, like: diabetes, systemic hypertension, chronic renal failure, rheumatoid arthritis, cardiac diseases and all of them had anemia.

Lesions were photographed for classification ^[2] and quantification of the wound area before and after application of LLLT, using software Image-Pro® Plus 6.0.

In this work, ulcer images were delimited in two regions of the lesion: PG1- outer margin (outside wound edge) and PG2- inner margin (inside edge wound). PG1 is total wound size, PG2 necrotic wound area. To calculate granulation

tissue (GT) area (scar tissue), the following equation was used: $GT = PG1 - PG2$. (granulation tissue is PG1 area minus PG2 area) Figure 1 illustrates evaluation method.

3mm² of granulation tissue (GT) were removed of each lesion in the first and last days after treatment. Samples were stored in RNA Lateral (Sigma) and kept at -80°C until they were used for qRT-PCR.

LLLT

Patients were placed on the bed and positioned in order to provide free access for LLLT of ulcerated area, wounds were irradiated once a day, for 12 consecutive days totalizing 12 applications.

Device: Thera Lase® (DMC, São Carlos – Brazil)

Parameters: 660nm wavelength (InGaAlP), 100mW power output, and energy density of 2J/cm². Laser emitter was positioned perpendicularly and applications were done around lesion area, 0.5cm distant from tissue to avoid contact between laser probe and irradiated points. Points were 2cm far from each other. Irradiation time at each point was 12 seconds.

qRT-PCR

Total mRNA was extracted from patient biopsy of 3mm² target tissue (peri-ulcerated tissue) using a commercial kit "Illustrates RNA spin Mini RNA Isolation Kit" (GE Healthcare) according to the manufacturer's instructions. Approximately 1µg of total RNA was used for cDNA synthesis tape (First-Strand cDNA Synthesis Kit®, GE Healthcare).

cDNA samples were subjected to trials of gene expression for IL-6, TNF-α, VEGF and TGF-β by Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) in the machine Stratagene Mx3005P, using the "Brilliant II SYBR® Green QPCR Master Mix" (Stratagene ®) with commercial primers (QuantiTect Primer Assay - Qiagen).

PCR amplification products were performed using the following ingredients: -15ng cDNA, 10mM forward primer, 10mM reverse primer, Syber Green (2X). Primers used were: IL-6 forward: 5'CTCAGCCCTGAGAAAGGAGA 3'; reverse: 5' TGATTTTCACCAAGGCAAGTCT 3'. TNF- α forward: 5'CTGCCCCAATCCCTTTATT 3'; reverse: 5'CCCAATTCTCTTTTGTAGCC 3'. VEGF forward: 5' CCTCCGAAACCATGAACTTT 3'; reverse: 5' GCAGTAGCTGCGCTGATAGA 3'. TGF- β forward: 5' GAGCCTGAGGCCGACTACTA 3'; reverse: 5' GGGTTCAGGTACCGCTTCTC 3'. Beta-actin: was used as an internal control (forward: AAGATTT GGCACCA CACTTTCTACA; reverse: CGGTGAGCAGCACAGGGT).

The reaction was completed with ultra-purified water, final volume of 20µl for each sample to be tested. Samples were pre-heated at 95°C for 10min. followed repeated 40 cycles of 95°C for 15sec., 60°C for 1 minute, 72°C to 15sec. one cycle for dissociation curve, as follows: 95°C for 1 minute, 55°C for 30 seconds, 95°C for 30sec.

The expression levels of target gene were then normalized to 18S ribosomal RNA. For data analysis we used the following formula: $-2\Delta Ct$ method of relative quantification ^[15]. The average values of cycle-threshold (CT) were used.

Statistical Analysis

For statistical analysis data were expressed as mean and SEM. Graph Pad Prism 5.0 software was used to ANOVA test, with post hoc of Tukey-Kramer. Significance level of 0.05%

Results

Macroscopically our results showed a reduction of wound size after LLLT (Figure 1)

Microscopically, after qRT-PCR, data indicated an improvement in the regeneration process:

Levels of VEGF increased after treatment. (Figure 2),

Levels of IL6 did not change after treatment. (Figure 3)

Levels of TNF- α reduced after treatment. (Figure 4)

Levels of TGF- β increased after treatment. (Figure 5)

<<Figure 1>>

<<Figure 2>>

<<Figure 3>>

<<Figure 4>>

<<Figure 5>>

Discussion

Wound healing process is divided into the following stages: inflammation, proliferation, epithelialization and remodeling, it is blocked or delayed when one of the steps is missing or out of sequence, this is often the case of PU.

At molecular level, failure of wound healing may result either in deficient or functional inhibition of growth factors ^[16] such as: IL6, TNF- α , VEGF, and TGF- β , analised in this study.

Despite the small number of patients, our findings related to incidence concluded that sacrococcygeal PS, one of the most common lesion^[17, 18], was also the highest incidence in this study, presenting NPUAP 2009, grade III or IV.

1
2
3
4 LLLT enhancement of wound healing in humans was first noticed at the pioneering studies of Mester and colleagues
5
6 in mid 80's. However, conflicting evidence stemmed from some recent and well designed experiments have failed to
7
8 corroborate this findings.
9

10 Mester et al (1985) performed an histological analysis of the effects of ruby laser with 694.3 nm in wound healing of
11
12 the back of mice. Laser was applied at a dose of 1.1 J/cm², twice a week for four sessions^[7]. LLLT (660 nm, 17
13
14 J/cm²) was applied in PU of paraplegic patients, twelve irradiations were done and after 8 weeks lesions were
15
16 healed^[19]. Our results observed a contraction of around 50% in the diameters of irradiated wounds after seven days
17
18 and also observed increased cellular activity at the edge and base of the wound, faster formation of granulation
19
20 tissue, and accelerated wound closure in irradiated group.
21

22 On the other hand, LLLT on venous leg ulcers daily for one month with HeNe using 1J/cm² and 4J/cm² ^[20], and
23
24 using red (632, 660, 685nm) and infrared (904, 810nm) no longer than a month, found no statistical differences
25
26 between groups^[21- 24], according to literature review that indicates LLLT does not improve wound healing in
27
28 humans^[25]. We credit that to difficulties comparing clinical trials due to different laser parameters, such as:
29
30 wavelength, number of sessions, energy density and ulcer origin.
31

32 LLLT over PU was studied mainly with infrared wavelength (820, 904nm). A prospective, observer-blinded
33
34 multicenter randomized clinical trial, using 1J/cm² five times a week for six weeks, found no statistical differences
35
36 between groups ^[26,27].
37

38 We used a diode laser (AsGaAl) in shorter wavelength (660nm), and doubled the energy density (2J/cm²) for shorter
39
40 periods, for twelve days, because energy density and exposure time are considered determinant to PS healing as
41
42 demonstrated in murine model ^[28].
43

44 Considering that most individuals in our study (62,5%) were diabetic, other studies, irradiating diabetic foot wounds
45
46 for three months with red diode (685nm) and 10J/cm², found that lasertherapy accelerated the healing process ^[29].
47
48 just like our findings they described an important reduction in granulation tissue and an increasing in the healing
49
50 rate^[30].
51

52 Wound healing is improved in conditions of reduced microcirculation ^[29]. In fact, blood flow recovering in skin
53
54 lesions was described in Egyptian diabetic patients by laser dopler flowmetry after LLLT ^[31].
55

56 Diabetic rats LLLT, (InGaAlP), power of 100mW, 660nm and energy density of 10J/cm², showed an increase in
57
58 collagen fibers, decreased macrophage production of granulation tissue, moderate amount of newly formed vessels,
59
60
61
62
63
64
65

and accelerated healing process. In our study, we found that LLLT over PU of diabetic patients increased factors involved in healing process that can accelerate wound healing ^[32], single case reports also support this LLLT healing enhancement ^[33,34].

Most of biochemical analyses of LLLT were carried out in animal models; but our study made that it in humans and our findings using qRT-PCR demonstrate higher levels of VEGF after LLLT, this is in agreement with other authors' findings, considering that VEGF is the most powerful mitogenic agent acting in endothelial cells and induction of vascular sprouting ^[35].

HeNe laser irradiation in continuous mode, 5mW power output, for 1, 2, 3, 5, 10, 15, 20, 30, 40 and 60 minutes, corroborate our findings increasing VEGF production in cell cultures of smooth muscle cells, fibroblasts and cardiac myocytes until 20 minutes exposure, after that time, there was a decrease in the production of this growth factor ^[35].

IL-6 is considered to participate in many events like inflammation, immune response, oncogenesis, cell growing and differentiation, in addition, IL-6 is elevated in diabetes ^[36], Cell cultures experiments shows modulation of IL-6 levels after laser irradiation ^[36, 37] and, animal models also indicate that after LLLT there was a reduction in the expression of IL-6 which may indicate a reduction of inflammation ^[38], but our analysis contrasted with them and didn't show differences of IL-6 levels after LLLT.

TNF- α is considered a pro-inflammatory cytokine. It is upregulated during the inflammatory phase of wound healing and is a powerful induction agent of interleukins production, reactive oxygen species generation and apoptosis. Diabetic patients and non-healing wounds have abnormally higher TNF- α level ^[36, 37].

Our results in human patients agree with cell culture findings, indicating a reduction of TNF- α levels ^[36, 37].

Diabetic individuals may exhibit overexpression of TNF- α and IL-6, which increases insulin resistance. In our study we have obtained a decrease in the expression of TNF- α after LLLT, which is associated to improvement in insulin sensitivity and endothelial function, improving vascular tone, platelet aggregation, coagulation and fibrinolysis, thereby improving the process of tissue repair ^[39].

Our experiment also demonstrate that after laser irradiation occurred an increase in expression of TGF- β , which is known to be the most potent growth factor involved in wound healing throughout the body, released by platelets at the site of injury. It may modulate inflammatory response, angiogenesis, epithelization, extracellular matrix deposition and remodeling ^[40, 41].

Conclusion

- LLLT promoted an improvement of the macroscopic appearance of PS grades III and IV.
- VEGF and TGF- β , growth factors related to tissue angiogenesis, increased after treatment.
- IL-6 did not change after treatment.
- TNF reduced after treatment.

In synthesis, LLLT is a promising healing tool to reduced PU size, after LLLT, wounds presented improvement in gross appearance, with increase in factors VEGF and TGF- β , which take part in angiogenesis, attract fibroblasts and stimulate collagen production.

References

- 1- Calil, J. A., Ferreira, L. M., Neto, M. S., CASTILHO, H. D., & Garcia, E. B. (2001). Clinical application of the VY posterior thigh fasciocutaneous flap. *Revista da Associação Médica Brasileira*, 47(4), 311-319.
- 2- National Pressure Ulcer Advisory Panel (2009) Classification of pressure ulcers. <http://www.npuap.org> Accessed: 10 august 2012.
- 3- Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *New England journal of medicine*, 341(10), 738-746.
- 4- Robson, M. C. (1997). The role of growth factors in the healing of chronic wounds. *Wound Repair and Regeneration*, 5(1), 12-17.
- 5- Trengove, N. J., Bielefeldt-Ohmann, H., & Stacey, M. C. (2000). Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers. *Wound Repair and Regeneration*, 8(1), 13-25.
- 6- de Braekt, M. M., van Alphen, F. A., Kuijpers-Jagtman, A. M., & Maltha, J. C. (1991). Effect of low level laser therapy on wound healing after palatal surgery in beagle dogs. *Lasers in surgery and medicine*, 11(5), 462-470.
- 7- Mester, E., Mester, A. F., & Mester, A. (1985). The biomedical effects of laser application. *Lasers in surgery and medicine*, 5(1), 31-39.
- 8- Yu, W., Naim, J. O., & Lanzafame, R. J. (1997). Effects of photostimulation on wound healing in diabetic mice. *Lasers in surgery and medicine*, 20(1), 56-63.
- 9- Maiya, G. A., Kumar, P., & Rao, L. (2005). Effect of low intensity helium-neon (He-Ne) laser irradiation on diabetic wound healing dynamics. *Photomedicine and Laser Therapy*, 23(2), 187-190.
- 10- Posten, W., Wrone, D. A., Dover, J. S., Arndt, K. A., Silapunt, S., & Alam, M. (2005). Low-level laser therapy for wound healing: mechanism and efficacy. *Dermatologic surgery*, 31(3), 334-340.
- 11- Viegas, V. N., Abreu, M. E. R., Viezzler, C., Machado, D. C., Filho, M. S. A., Silva, D. N., & Pagnoncelli, R. M. (2007). Effect of low-level laser therapy on inflammatory reactions during wound healing: comparison with meloxicam. *Photomedicine and laser surgery*, 25(6), 467-473.
- 12- Huang, Y. Y., Chen, A. C. H., Carroll, J. D., & Hamblin, M. R. (2009). Biphasic dose response in low level light therapy. *Dose-Response*, 7(4), dose-response.
- 13- Saygun, I., Nizam, N., Ural, A. U., Serdar, M. A., Avcu, F., & Tözüm, T. F. (2012). Low-level laser irradiation affects the release of basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and receptor of IGF-I (IGFBP3) from osteoblasts. *Photomedicine and laser surgery*, 30(3), 149-154.
- 14- Dang, Y., Liu, B., Liu, L., Ye, X., Bi, X., Zhang, Y., & Gu, J. (2011). The 800-nm diode laser irradiation induces skin collagen synthesis by stimulating TGF- β /Smad signaling pathway. *Lasers in medical science*, 26(6), 837-843.
- 15- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *methods*, 25(4), 402-408.
- 16- Wang, C. J., Ko, J. Y., Kuo, Y. R., & Yang, Y. J. (2011). Molecular changes in diabetic foot ulcers. *Diabetes research and clinical practice*, 94(1), 105-110.
- 17- Rocha, J., Miranda, M., & Andrade, M. (2006). [Pressure ulcer management--Evidence-based interventions]. *Acta Médica Portuguesa*, 29-38.

- 18- Costa, M. P., Sturtz, G., Costa, F. P. P. D., Ferreira, M. C., & Barros Filho, T. E. (2005). Epidemiological profile and treatment of pressure ulcers: experience with 77 cases. *Acta Ortopédica Brasileira*, 13(3), 124-133.
- 19- Lopes, L. D. F. Utilização do laser de 660 nm, 17 J/cm² em úlceras por pressão– Um relato de caso.
- 20- Santoianni, P., Monfrecola, G., Martellotta, D., & Ayala, F. (1984). Inadequate effect of helium-neon laser on venous leg ulcers. *Photo-dermatology*, 1(5), 245-249.
- 21- Malm, M., & Lundeberg, T. (1991). Effect of low power gallium arsenide laser on healing of venous ulcers. *Scandinavian journal of plastic and reconstructive surgery and hand surgery*, 25(3), 249-251.
- 22- Lundeberg, T., & Malm, M. (1991). Low-power HeNe laser treatment of venous leg ulcers. *Annals of plastic surgery*, 27(6), 537-539.
- 23- Gupta, A. K., Filonenko, N., Salansky, N., & Sauder, D. N. (1998). The Use of Low Energy Photon Therapy (LEPT) in Venous Leg Ulcers: A Double-Blind, Placebo-Controlled Study. *Dermatologic Surgery*, 24(12), 1383-1386.
- 24- Kirtschig, G., Brink, A., Scheper, R., & Jaspars, E. (2005). Low level laser: does it influence wound healing in venous leg ulcers? A randomized, placebo-controlled, double-blind study. *J Clin Microbiol*, 40, 779-87.
- 25- Sobanko, J. F., & Alster, T. S. (2008). Efficacy of Low-Level Laser Therapy for Chronic Cutaneous Ulceration in Humans: A Review and Discussion. *Dermatologic surgery*, 34(8), 991-1000.
- 26- Nussbaum, E. L., Biemann, I., & Mustard, B. (1994). Comparison of ultrasound/ultraviolet-C and laser for treatment of pressure ulcers in patients with spinal cord injury. *Physical therapy*, 74(9), 812-823.
- 27- Lucas, C., Van Gemert, M. J. C., & De Haan, R. J. (2003). Efficacy of low-level laser therapy in the management of stage III decubitus ulcers: a prospective, observer-blinded multicentre randomised clinical trial. *Lasers in medical science*, 18(2), 72-77.
- 28- Lanzafame, R. J., Stadler, I., Kurtz, A. F., Connelly, R., Brondon, P., & Olson, D. (2007). Reciprocity of exposure time and irradiance on energy density during photoradiation on wound healing in a murine pressure ulcer model. *Lasers in surgery and medicine*, 39(6), 534-542.
- 29- Kaviani, A., Djavid, G. E., Ataie-Fashtami, L., Fateh, M., Ghodsi, M., Salami, M., Zand N., Kashef N., Larijani, B. (2011). A randomized clinical trial on the effect of low-level laser therapy on chronic diabetic foot wound healing: a preliminary report. *Photomedicine and Laser Surgery*, 29(2), 109-114.
- 30- Minatel, D. G., Frade, M. A. C., França, S. C., & Enwemeka, C. S. (2009). Phototherapy promotes healing of chronic diabetic leg ulcers that failed to respond to other therapies. *Lasers in surgery and medicine*, 41(6), 433-441.
- 31- Saied, G. M., Kamel, R. M., Labib, A. M., Said, M. T., & Mohamed, A. Z. (2011). The diabetic foot and leg: combined He-Ne and infrared low-intensity lasers improve skin blood perfusion and prevent potential complications. A prospective study on 30 Egyptian patients. *Lasers in medical science*, 26(5), 627-632.
- 32- Carvalho, P. D. T. C. D., Silva, I. S. D., Reis, F. A. D., Perreira, D. M., & Aydos, R. D. (2010). Influence of ingaalp laser (660nm) on the healing of skin wounds in diabetic rats. *Acta Cirúrgica Brasileira*, 25(1), 71-79.
- 33- Dixit, S., Maiya, A., Umakanth, S., & Borkar, S. (2013). Photobiomodulation of surgical wound dehiscence in a diabetic individual by low-level laser therapy following median sternotomy. *Indian journal of palliative care*, 19(1), 71.
- 34- Dixit, S., Agrawal, P. R., Sharma, D. K., & Singh, R. P. (2014). Closure of chronic non healing ankle ulcer with low level laser therapy in a patient presenting with thalassemia intermedia: Case report. *Indian journal of plastic surgery: official publication of the Association of Plastic Surgeons of India*, 47(3), 432.
- 35- Kipshidze, N., Nikolaychik, V., Keelan, M. H., Shankar, L. R., Khanna, A., Kornowski, R., ... & Moses, J. (2001). Low-power helium: Neon laser irradiation enhances production of vascular endothelial growth factor and promotes growth of endothelial cells in vitro. *Lasers in surgery and medicine*, 28(4), 355-364.
- 36- Sekhejane, P. R., Houreld, N. N., & Abrahamse, H. (2011). Irradiation at 636 nm positively affects diabetic wounded and hypoxic cells in vitro. *Photomedicine and laser surgery*, 29(8), 521-530.
- 37- Houreld, N. N., Sekhejane, P. R., & Abrahamse, H. (2010). Irradiation at 830 nm stimulates nitric oxide production and inhibits pro-inflammatory cytokines in diabetic wounded fibroblast cells. *Lasers in surgery and medicine*, 42(6), 494-502.
- 38- Pallotta, R. C., Bjordal, J. M., Frigo, L., Junior, E. C. P. L., Teixeira, S., Marcos, R. L., ... & Lopes-Martins, R. Á. B. (2012). Infrared (810-nm) low-level laser therapy on rat experimental knee inflammation. *Lasers in medical science*, 27(1), 71-78.

- 1
2
3
4 39- Carvalho, M. H. C. D., Colaço, A. L., & Fortes, Z. B. (2006). Cytokines, endothelial dysfunction, and
5 insulin resistance. *Arquivos Brasileiros de Endocrinologia & Metabologia*, 50(2), 304-312.
6 40- Tonnesen, M. G., Feng, X., & Clark, R. A. (2000, December). Angiogenesis in wound healing. In *Journal*
7 *of Investigative Dermatology Symposium Proceedings* (Vol. 5, No. 1, pp. 40-46). Nature Publishing Group.
8 41- Riedel, K., Riedel, F., Goessler, U. R., Germann, G., & Sauerbier, M. (2007). TGF- β Antisense Therapy
9 Increases Angiogenic Potential in Human Keratinocytes In Vitro*. *Archives of medical research*, 38(1), 45-
10 51.
11

12 Captions

13
14
15 **Figure 1-** Example of Image-Pro ® Plus 6.0 software measuring the lumbosacral wound area. PG1 external wound
16 measurement and PG2 internal wound area measurement, granulation tissue size (GT) is PG1 minus PG2.
17

18
19 **Figure 2-** Graphic comparing VEGF gene-expression (qRT-PCR) before and after LLLT, VEGF/18S before LLLT
20 (Pre) 1,0+- 0,19; after (Pos) 1,47 +- 0,42, * $P<0,05$.
21

22
23 **Figure 3-** Graphic comparing IL-6 gene-expression (qRT-PCR) before and after laser therapy. IL6/18S before
24 lasertherapy (Pre) 1,0 +- 0,23 ; after (Pos) 1,01+-0,47, * $P<0,05$.
25

26
27 **Figure 4-** Graphic comparing TNF- α gene-expression (qRT-PCR) before and after laser therapy. TNF/18S before
28 lasertherapy (Pre) 1,45 +- 0,36; after (Pos) 0,84 +- 0,23, * $P<0,05$.
29

30
31 **Figure 5** Graph comparing TGF- β gene-expression (qRT-PCR) before and after laser therapy. TGF/18S before
32 lasertherapy (Pre) 1,0 +-0,88; after (Pos) 4,0+-0,12, * $P<0,05$.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Laser-photobiomodulation in human pressure ulcer healing: gene-expression analysis of inflammatory biochemical markers.

Anelice Calixto Ruh¹, Lúcio Frigo³, Marcos Fernando Xisto Braga Cavalcanti^{3,6}, Paulo Svidnicki², Viviane Nogaroto Vicari², Rodrigo Alvaro Brandão Lopes-Martins^{4,5}, Ernesto Cesar Pinto Leal Junior⁴, Natalia De Isla⁶, Francesca Diomedè⁷, Oriana Trubiani⁷, Giovani Marino Favero¹

1 – Biological and Health Science, Multidisciplinary Laboratory, State University of Ponta Grossa (UEPG), Ponta Grossa, PR, Brazil, Av. Gal. Carlos Cavalcanti, 4748 Ponta Grossa 84030-900, PR – Brazil, anelicecalixto@yahoo.com.br, gmfavero@yahoo.com.br

2 - Departamento de Biologia Estrutural, Molecular e Genética, State University of Ponta Grossa (UEPG), Av. Carlos Cavalcanti, 4748, Ponta Grossa, PR 84030–900, Brazil, paulovini_pg@hotmail.com, vivianenogaroto@hotmail.com

3 – Laser in Dentistry Program, Cruzeiro do Sul University (UNICSUL). Av. Dr. Ussiel Cirilo, 225 São Miguel Paulista, 08060-070 São Paulo, SP – Brazil, Rua Galvão Bueno 868, Liberdade, São Paulo, SP, Brasil, Cep 01506-000 - Phone: +55 11 33853000 fax: +55 11 33853000, luciofrigo@uol.com.br, mxistocavalcanti@gmail.com

4 – Center for Research and Innovation in Laser, Nove de Julho University (UNINOVE), Rua Vergueiro 235, São Paulo 01504-001, SP – Brazil. ernesto.leal.junior@gmail.com

5 – Technological Innovation Center, Mogi das Cruzes University. Av. Candido Almeida de Xavier e Souza, 200, Mogi das Cruzes, 08780-911, SP – Brazil. ralopesmartins@gmail.com

6 - UMR 7365 CNRS-Université de Lorraine, Faculté de Médecine - 9, avenue de la Forêt de Haye, Vandoeuvre-lès-Nancy, France, cep 54500, phone: [+33 \(0\)3.83.68.36.84](tel:+3323383683684), fax: [+33 \(0\)3.83.68.36.89](tel:+3323383683689), email : natalia.de-isla@univ-lorraine.fr.

7 - Department of Medical, Oral and Biotechnological Sciences, “G. d’Annunzio” University, Chieti-Pescara, 66100 Chieti, Italy, phone and fax: +37 08713551, email: francesca.diomedè@unich.it, trubiani@unich.it.

Corresponding author: Marcos Fernando Xisto Braga Cavalcanti - mxistocavalcanti@gmail.com - Rua Galvão Bueno 868, Liberdade, São Paulo, SP, Brasil, Cep 01506-000 - Phone: +55 11 33853000 fax: +55 11 33853000.









