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

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Lasers in Medical Science

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

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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 (InGaAlP, 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

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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]

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4 The effects of laser radiation over living tissues are based on the absorption of its energy and its transduction into a
5 biological process, mainly due ATP synthesis ^[12]. In addition, LLLT may modulate transcription of several growth
6 factors, including FGF and VEGF. ^[13, 14]
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10 In this perspective, the aim of this study was evaluating the effects of LLLT on macroscopic aspect and mRNA
11 expression of genes related to wound healing in diabetic patients presenting pressure ulcers.
12
13

14 **Materials and Methods**

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16 Research Ethics Committee of Ponta Grossa University (COEP-UEPG) approved this study (protocol number
17 07897/11, opinion 48/2011), that was performed in accordance with the ethical standards as laid down in the 1964
18 Declaration of Helsinki and its later amendments or comparable ethical standards.
19
20

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

22
23 Eight volunteers, patients at Santa Casa de Misericordia Hospital at Ponta Grossa (SCMPG), met the following
24 inclusion criteria: age between 30 to 75 years old, diabetic, pressure ulcer (PU) presenting grade II, III or IV,
25 NPUAP ^[2].
26
27

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29 According to NPUAP 2009 ^[2], PS grade III shows significant skin loss involving damage or necrosis of
30 subcutaneous tissue that may extend to the fascia. In grade IV PS a significant loss occurs with extensive destruction
31 of skin and necrosis of subcutaneous tissue and/or muscle, bone or supporting structures like tendons and joint
32 capsules.
33
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38 **Sample**

39
40 Seven male, one female, average age was 62.5 years, one calcaneus PU grade III and seven PU sacrococcygeal
41 grade III or IV. All subjects in the study were hospitalized in wards or intensive care unit, immobilized in bed, due
42 to primary diseases.
43
44

45 All were volunteers and had different kinds of diseases, like: diabetes, systemic hypertension, chronic renal failure,
46 rheumatoid arthritis, cardiac diseases and all of them had anemia.
47
48
49

50 Lesions were photographed for classification ^[2] and quantification of the wound area before and after application of
51 LLLT, using software Image-Pro[®] Plus 6.0.
52
53

54 In this work, ulcer images were delimited in two regions of the lesion: PG1- outer margin (outside wound edge) and
55 PG2- inner margin (inside edge wound). PG1 is total wound size, PG2 necrotic wound area. To calculate granulation
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4 tissue (GT) area (scar tissue), the following equation was used: $GT = PG1 - PG2$. (granulation tissue is PG1 area
5 minus PG2 area) Figure 1 illustrates evaluation method.
6

7
8 3mm² of granulation tissue (GT) were removed of each lesion in the first and last days after treatment. Samples were
9
10 stored in RNA Lateral (Sigma) and kept at -80°C until they were used for qRT-PCR.
11

12 **LLLT**

13
14 Patients were placed on the bed and positioned in order to provide free access for LLLT of ulcerated area, wounds
15
16 were irradiated once a day, for 12 consecutive days totalizing 12 applications.
17

18
19 Device: Thera Lase® (DMC, São Carlos – Brazil)
20

21 Parameters: 660nm wavelength (InGaAlP), 100mW power output, and energy density of 2J/cm². Laser emitter was
22
23 positioned perpendicularly and applications were done around lesion area, 0.5cm distant from tissue to avoid contact
24
25 between laser probe and irradiated points. Points were 2cm far from each other. Irradiation time at each point was 12
26
27 seconds.
28

29 **qRT-PCR**

30
31 Total mRNA was extracted from patient biopsy of 3mm² target tissue (peri-ulcerated tissue) using a commercial kit
32
33 "Illustrates RNA spin Mini RNA Isolation Kit" (GE Healthcare) according to the manufacturer's instructions.
34
35 Approximately 1µg of total RNA was used for cDNA synthesis tape (First-Strand cDNA Synthesis Kit®, GE
36
37 Healthcare).
38

39
40 cDNA samples were subjected to trials of gene expression for IL-6, TNF-α, VEGF and TGF-β by Quantitative Real
41
42 Time Polimerase Chain Reaction (qRT-PCR) in the machine Stratagene Mx3005P, using the "Brilliant II SYBR®
43
44 Green QPCR Master Mix" (Stratagene ®) with commercial primers (Quatin Tect Primer Assay - Qiagen).
45

46 PCR amplification products were performed using the following ingredients: -15ng cDNA, 10mM forward primer,
47
48 10mM reverse primer, Syber Green (2X). Primers used were: IL-6 forward: 5'CTCAGCCCTGAGAAAGGAGA 3';
49
50 reverse: 5' TGATTTTCACCAGGCAAGTCT 3'. TNF- α forward: 5'CTGCCCAATCCCTTTATT 3'; reverse:
51
52 5'CCCAATTCTCTTTTTGAGCC 3'. VEGF forward: 5' CCTCCGAAACCATGAACTTT 3'; reverse: 5'
53
54 GCAGTAGCTGCGCTGATAGA 3'. TGF- β forward: 5' GAGCCTGAGGCCGACTACTA 3'; reverse: 5'
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56 GGGTTCAGGTACCGCTTCTC 3'. Beta-actin: was used as an internal control (forward: AAGATTT GGCACCA
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58 CACTTTCTACA; reverse: CGGTGAGCAGCACAGGGT).
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4 The reaction was completed with ultra-purified water, final volume of 20µl for each sample to be tested. Samples
5 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
6
7
8 15sec. one cycle for dissociation curve, as follows: 95°C for 1 minute, 55°C for 30 seconds, 95°C for 30sec.
9

10 The expression levels of target gene were then normalized to 18S ribosomal RNA. For data analysis we used the
11 following formula: $-2\Delta Ct$ method of relative quantification ^[15]. The average values of cycle-threshold (CT) were
12
13 used.
14

15 **Statistical Analysis**

16
17 For statistical analysis data were expressed as mean and SEM. Graph Pad Prism 5.0 software was used to ANOVA
18
19 test, with post hoc of Tukey-Kramer. Significance level of 0.05%
20
21

22 **Results**

23
24 Macroscopically our results showed a reduction of wound size after LLLT (Figure 1)

25
26 Microscopically, after qRT-PCR, data indicated an improvement in the regeneration process:

27
28 Levels of VEGF increased after treatment. (Figure 2),

29
30 Levels of IL6 did not change after treatment. (Figure 3)

31
32 Levels of TNF- α reduced after treatment. (Figure 4)

33
34 Levels of TGF- β increased after treatment. (Figure 5)

35
36 <<Figure 1>>

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38 <<Figure 2>>

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40 <<Figure 3>>

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42 <<Figure 4>>

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44 <<Figure 5>>

45 **Discussion**

46
47 Wound healing process is divided into the following stages: inflammation, proliferation, epithelialization and
48
49 remodeling, it is blocked or delayed when one of the steps is missing or out of sequence, this is often the case of PU.
50

51 At molecular level, failure of wound healing may result either in deficient or functional inhibition of growth factors
52
53 ^[16] such as: IL6, TNF- α , VEGF, and TGF- β , analised in this study.
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57 Despite the small number of patients, our findings related to incidence concluded that sacrococcygeal PS, one of the
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59 most common lesion^[17, 18], was also the highest incidence in this study, presenting NPUAP 2009, grade III or IV.
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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
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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
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58 collagen fibers, decreased macrophage production of granulation tissue, moderate amount of newly formed vessels,
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4 and accelerated healing process. In our study, we found that LLLT over PU of diabetic patients increased factors
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6 involved in healing process that can accelerate wound healing ^[32], single case reports also support this LLLT healing
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8 enhancement ^[33,34].
9

10 Most of biochemical analyses of LLLT were carried out in animal models; but our study made that it in humans and
11
12 our findings using qRT-PCR demonstrate higher levels of VEGF after LLLT, this is in agreement with other
13
14 authors' findings, considering that VEGF is the most powerful mitogenic agent acting in endothelial cells and
15
16 induction of vascular sprouting ^[35].
17

18 HeNe laser irradiation in continuous mode, 5mW power output, for 1, 2, 3, 5, 10, 15, 20, 30, 40 and 60 minutes,
19
20 corroborate our findings increasing VEGF production in cell cultures of smooth muscle cells, fibroblasts and cardiac
21
22 myocytes until 20 minutes exposure, after that time, there was a decrease in the production of this growth factor ^[35].
23

24 IL-6 is considered to participate in many events like inflammation, immune response, oncogenesis, cell growing and
25
26 differentiation, in addition, IL-6 is elevated in diabetes ^[36], Cell cultures experiments shows modulation of IL-6
27
28 levels after laser irradiation ^[36, 37] and, animal models also indicate that after LLLT there was a reduction in the
29
30 expression of IL-6 which may indicate a reduction of inflammation ^[38], but our analysis contrasted with them and
31
32 didn't show differences of IL-6 levels after LLLT.
33

34
35 TNF- α is considered a pro-inflammatory cytokine. It is upregulated during the inflammatory phase of wound healing
36
37 and is a powerful induction agent of interleukins production, reactive oxygen species generation and apoptosis.
38
39 Diabetic patients and non-healing wounds have abnormally higher TNF- α level ^[36, 37].
40

41 Our results in human patients agree with cell culture findings, indicating a reduction of TNF- α levels ^[36, 37].
42

43 Diabetic individuals may exhibit overexpression of TNF- α and IL-6, which increases insulin resistance. In our study
44
45 we have obtained a decrease in the expression of TNF- α after LLLT, which is associated to improvement in insulin
46
47 sensitivity and endothelial function, improving vascular tone, platelet aggregation, coagulation and fibrinolysis,
48
49 thereby improving the process of tissue repair ^[39].
50

51 Our experiment also demonstrate that after laser irradiation occurred an increase in expression of TGF- β , which is
52
53 known to be the most potent growth factor involved in wound healing throughout the body, released by platelets at
54
55 the site of injury. It may modulate inflammatory response, angiogenesis, epithelization, extracellular matrix
56
57 deposition and remodeling ^[40, 41].
58

59 **Conclusion**

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- 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.

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12 Captions

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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.
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19 **Figure 2-** Graphic comparing VEGF gene-expression (qRT-PCR) before and after LLLT, VEGF/18S before LLLT
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21 **(Pre)** 1,0+- 0,19; after **(Pos)** 1,47 +- 0,42, * $P < 0,05$.
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24 **Figure 3-** Graphic comparing IL-6 gene-expression (qRT-PCR) before and after laser therapy. IL6/18S before
25 lasertherapy **(Pre)** 1,0 +- 0,23 ; after **(Pos)** 1,01+-0,47, * $P < 0,05$.
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28 **Figure 4-** Graphic comparing TNF- α gene-expression (qRT-PCR) before and after laser therapy. TNF/18S before
29 lasertherapy **(Pre)** 1,45 +- 0,36; after **(Pos)** 0,84 +- 0,23, * $P < 0,05$.
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32 **Figure 5** Graph comparing TGF- β gene-expression (qRT-PCR) before and after laser therapy. TGF/18S before
33 lasertherapy **(Pre)** 1,0 +-0,88; after **(Pos)** 4,0+-0,12, * $P < 0,05$.
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Laser-photobiomodulation in human pressure ulcer healing: gene-expression analysis of inflammatory biochemical markers.

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