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Diane Duarte Hartmann

EFEITOS DO *LASER* SOBRE AS ALTERAÇÕES BIOQUÍMICAS E MORFOLÓGICAS INDUZIDAS POR UM MODELO DE LESÃO CUTÂNEA EM RATOS

Santa Maria, RS.

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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

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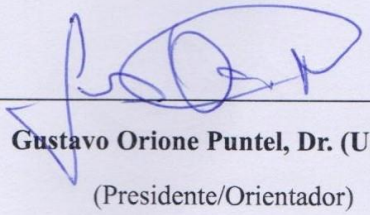

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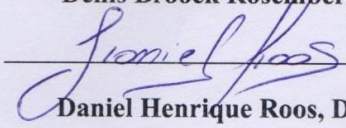

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DEDICATÓRIA

Dedico essa dissertação aos meus pais, Carlos e Ivani Hartmann.

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APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas trabalhados nesta dissertação.

A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados no item MANUSCRITO sob a forma de um manuscrito redigido em inglês conforme as normas do periódico Lasers Medical Science, ao qual será submetido. No mesmo constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final desta dissertação, apresentam conclusões gerais sobre os resultados do manuscrito presente neste trabalho e as perspectivas para futuros trabalhos.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO desta dissertação**.**

RESUMO

EFEITOS DO *LASER* SOBRE AS ALTERAÇÕES BIOQUÍMICAS E MORFOLÓGICAS INDUZIDAS POR UM MODELO DE LESÃO CUTÂNEA EM RATOS

AUTORA: Diane Duarte Hartmann
ORIENTADOR: Prof. Dr. Gustavo Orione Puntel

A úlcera cutânea é uma patologia que se não tratada adequadamente pode levar a sérias complicações. Entre os recursos utilizados para o tratamento destas lesões de pele está a terapia a *laser* de baixa intensidade (LBI), a qual é amplamente estudada na literatura, visando facilitar/agilizar/e acelerar a cicatrização tecidual. Mas ainda pouco se sabe desse método terapêutico e o estresse oxidativo. Para melhor elucidar, este estudo teve como objetivo avaliar os efeitos do LBI sobre as alterações hematológicas e alterações bioquímicas na pele, como o estresse oxidativo, capacidade antioxidante, atividade das desidrogenases mitocondriais, e alterações morfológicas induzidas por um modelo de lesão cutânea em ratos, feitas através do instrumento cirúrgico "*punch*" em diferentes períodos de tempo,correlacionados as fases do processos inflamatório e cicatricial. Para a realização deste estudo foram utilizados 70 ratos da raça Wistar, divididos em grupo Controle, Sham, Lesão e Lesão+LLLT. As análises experimentais bioquímicas e morfológicas foram realizadas em diferentes momentos no 1º, 3º, 7º, 14º, 21º dia após o desenvolvimento da lesão, a partir de amostras de tecidos cutâneos, e amostra sanguínea, a fim de investigar a evolução cronológica das lesões bem como verificar a eficácia da terapia a *laser* empregada. O grupo lesionado e tratado demonstrou um aumento no número total de leucócitos, os níveis de DCF reduzida em comparação com os valores observados mo 1º dia e reduziu os níveis de TBARS no 7º até o 21º dia, quando comparados ao grupo controle. Os níveis de redução de MTT e níveis de SH não-proteíco apresentaram um aumentado no 1º dia após a lesão da pele com o tratamento com LBI. Considerando os dados da LBI acelerou a reparação dos tecidos da pele em um mecanismo que envolve aumentos de produção ROS no estágio inicial da cicatrização do tecido.

Palavras chaves: LBI, lesão cutânea, inflamação, cicatrização tecidual.

ABSTRACT

EFFECTS OF LASER ON BIOCHEMICAL AND MORPHOLOGICAL ALTERATIONS INDUCED A MODEL OF SKIN INJURY IN RATS

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ADVISOR: Gustavo Orione Puntel

Skin ulcer is a condition that can lead to serious complications, when not treated. Among the resources used to treat skin lesions is the low level laser therapy (LLLT), is extensively studied in the literature, wich could accelerate tissue healing. Thus, this study aims to evaluate the effects of LLLT on the hematological changes and biochemical alterations in the skin, such as oxidative stress, antioxidant capacity, mitochondrial dehydrogenase activity and morphological changes induced by a skin injury model in rats, made through the instrument surgical "punch". For these studies were used 70 Wistar rats that were divided into Control, Sham, Lesion and Lesion+LLLT groups. The biochemical and morphological experimental analyzes were performed at different times in 1st, 3rd, 7th, 14th, 21st day after the development of injury from skin tissue samples and blood sample in order to investigate the chronological development of the well lesions how to check the effectiveness of laser therapy used. Lesion+LLLT group showed an increase in total number of leukocytes, reduced DCF levels in comparison to values observed in 1 day and reduced the TBARS levels in 7th to 21th days, when compared to control group. The MTT reduction levels and NPSH levels were increased in the 1st day after skin injury with treatment group. Considering data the LLLT accelerated skin tissue repair in a mechanism that involves increases of ROS production at early stages of tissue healing.

Key words: LLLT, skin lesions, inflammation, tissue healing.

LISTA DE ILUSTRAÇÕES

Introdução

Manuscrito

LISTA DE ABREVIATURAS

Introdução

ET-1: Endotelina-1
NO: Óxido nítrico
PGI-2: Prostaciclina
ERO: Espécies reativas de oxigênio
O_2^-: Ânion superóxido
H_2O_2: Peróxido de hidrogênio
(HO•): radical hidroxila
ER: Espécies reativas
ERN: Espécies reativas de nitrogênio
$ONOO^-$: Peroxinitrito
SOD: Superóxido dismutase
CAT: Catalase
GSH: Glutationa reduzida
PDGF: Fator de crescimento derivado de plaquetas
TGF-β: Fator de crescimento transformante β
TGF-α: Fator de crescimento transformante α
EGF: Fator de crescimento epidérmico
VEGF: Fator de crescimento de células endoteliais
LBI: Laser de baixa intensidade
ATP: Adenosina trifosfato
TBARS: Substâncias reativas ao ácido tiobarbitúrico
DCFH-RS: Diclorofluoresceina reduzida
-SH: Grupos tióis não protéicos

Manuscrito

ROS: Reactive oxygen species
Al-Ga-In-P: Aluminium-Gallium-Indium-Phosphide
TP: Total protein
TG: Total globulin
WBC: Total leukocytes
RBC: Total erythrocytes
Ht: Hematocrit
Hb: Hemoglobin concentration
MCV: Mean corpuscular volume
MCHC: Mean corpuscular hemoglobin concentration
MDA: Malondiadehyde
TBA: Thibarbituric acid
TBARS: Thiobarbituric acid reactive substances
H_2DCF-DA: Reduced dichlorofluoresceine
DCF: Oxidized diclorofluoresceine
MTT: Methyl tetrazolium
DMSO: Dimethyl sulphoxide
SOD: Superoxide dismutase
CAT: Catalase
NPSH: Non-protein thiol
GSH: Glutathione reduced
BSA: Bovine serum albumin
HeNe: Helium-Neônio
LPS: Lipopolysaccharide
HeLa: Human cervical cancer cells
MMP: Membrane mitochondrial potential
O_2^-: Superoxide anion

SUMÁRIO

1. INTRODUÇÃO

1.1. ÚLCERAS CUTÂNEAS

O termo úlceras cutâneas é usado para nomear lesões que acometem a integridade da pele (Frade *et al.*, 2012). Essas são classificadas pela sua etiologia, sendo definidas em úlceras venosas, arteriais, associadas a neuropatias, e as úlceras por pressão(Phillips, 1994).

No Brasil, estima-se que cerca de 1,5 milhões de pessoas são diagnosticas com úlceras em membros inferiores (Siqueira *et al.*, 2009). Nos EUA 60% de todas as amputações não-traumáticas de extremidades inferiores estão associadas a úlceras diabéticas (Control e Prevention, 2011). Dentre as complicações da falta de tratamento adequado além das amputações, as úlceras podem levar ao atraso na reabilitação de enfermidades, e quando associadas com outras enfermidades podem levar até mesmo à morte do paciente (Ledoux *et al.*, 2013; Liao *et al.*, 2013).

A associação de fatores, tais como a mobilidade limitada, má perfusão, e estado de pele alterada (incluindo incidência prévia de úlceras de pressão, vermelhidão e presença de umidade) são preditores do desenvolvimento de úlceras (Madden, 2015). Além disso, idade, sexo, estado nutricional e doenças crônicas são outros fatores que proporcionam o aparecimento de úlceras, podendo assim estar relacionados com o nível de consciência, percepção sensorial e também com o prejuízo no sistema imunológico dos indivíduos (Campos *et al.*, 2010).

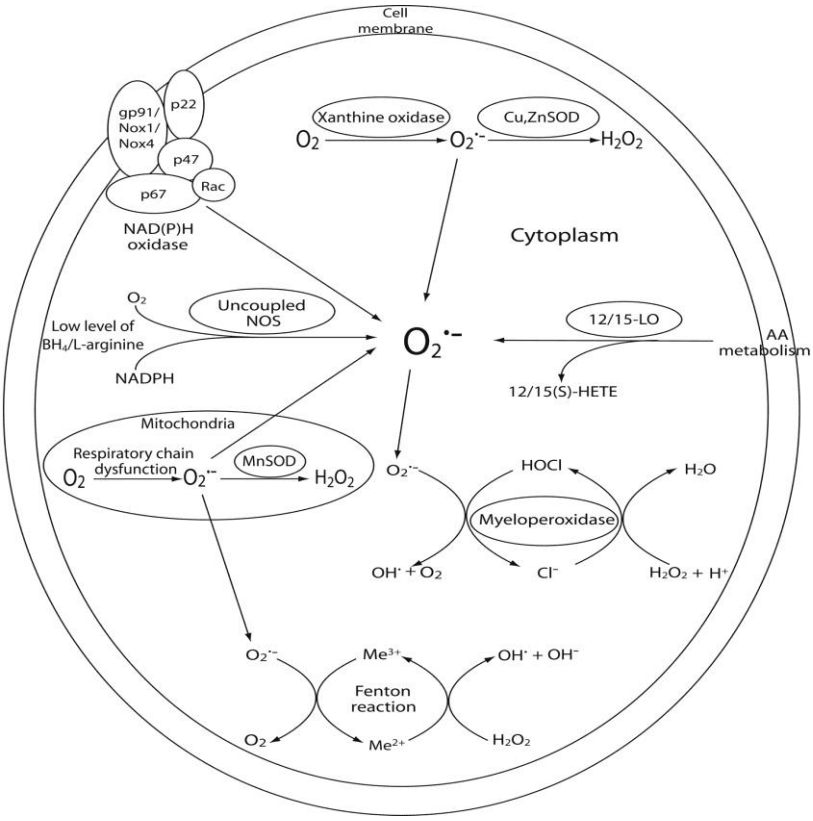
Mudanças estruturais nos capilares sanguíneos são uma das principais alterações fisiopatológicas determinadas pelas úlceras cutâneas, que provocam episódios de isquemia e reperfusão tecidual. O endotélio vascular lesado aumenta a produção de fatores de vasoconstrição e diminui fatores de vasodilatação, como endotelina-1 (ET-1), óxido nítrico (NO) e prostaciclina-2 (PGI-2) (Leroy e Silver, 1996; Corazza *et al.*, 2007; Michaelis *et al.*, 2012).

1.1.1 Estresse oxidativo

Alterações na perfusão tecidual como anóxia causada pela isquemia e a reperfusão podem induzir perturbações na homeostase celular (Cury *et al.*, 2013), e alterações na pressão oncótica do plasma, assim como o aumento geração de espécies reativas oxigênio (ERO)

(Júnior *et al.*, 2005; Corazza *et al.*, 2007; Francischetti *et al.*, 2010). Além disso, o processo de fosforilação oxidativa mitocondrial, é a principal forma de produção de ERO na pele, como o demonstrado no estudo de Anderson e colaboradores (2014). Outros estudos que utilizaram cultura de células da pele e mitocôndrias isoladas mostraram um aumento da produção do ânion superóxido a partir do vazamento de elétrons a partir da cadeia respiratória (Aitken *et al.*, 2007; Henderson *et al.*, 2009). Além do ânion superóxido (O_2^-), existem outras espécies reativas de oxigênio formadas pela redução parcial do oxigênio, como peróxido de hidrogênio (H_2O_2), e o radical hidroxila (HO•) (Ray *et al.*, 2012) (fig 1).

Figura 1 – Fontes geradoras de espécies reativas de oxigênio

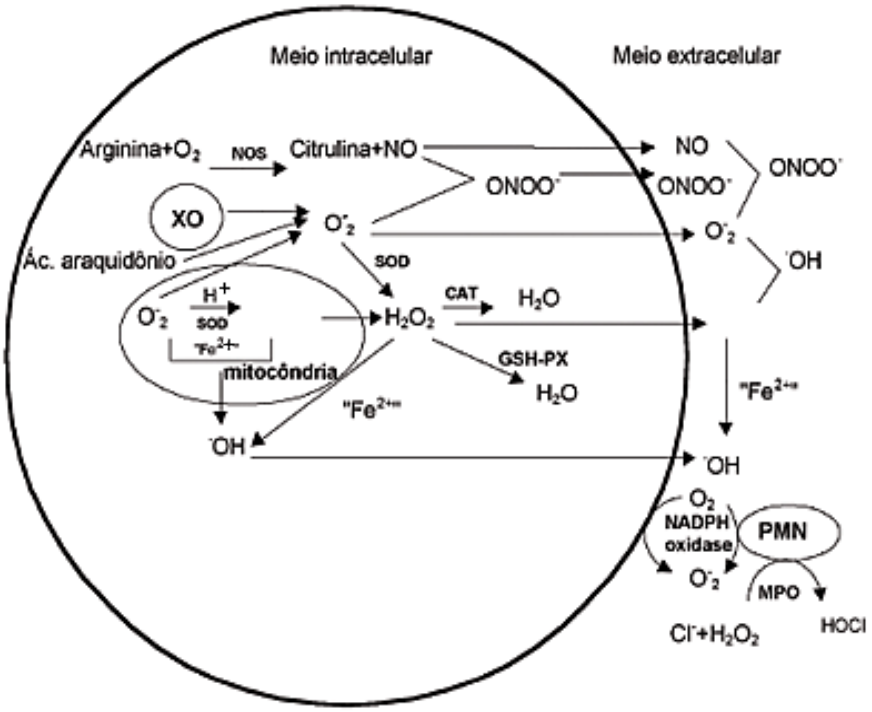


Fonte: (Madamanchi *et al.*, 2005).

Além disso, a interação do NO com outras espécies reativas (ER) promove a geração de espécies reativas de nitrogênio (ERN) (fig. 2). O representante principal de ERNs é peroxinitrito ($ONOO^-$), que pode ser formado a partir de ânion superóxido e NO (Kammeyer e

Luiten, 2015). Existem também espécies reativas derivadas cloro, ferro e enxofre (Halliwell, 2011; Radomska-Leśniewska *et al.*, 2016).

Figura 2 - Vias de formação de EROs e ERNs



Fonte: (Silveira, 2004)

A isquemia e reperfusão, assim como a produção de ER, e a resposta inflamatória estão diretamente relacionadas às úlceras cutâneas. Sendo assim, a resposta inflamatória exacerbada, está diretamente associado a um aumento excessivo na produção de ER (Saito *et al.*, 2008). Este dano oxidativo é capaz de gerar citotoxicidade assim como, um aumento na resposta inflamatória (Vaya, 2013). Estudos apontam que o aumento da enzima superóxido dismutase e a consequente redução dos níveis de ERO, promove uma redução da resposta inflamatória (Gao *et al.*, 2008).

O adequado equilíbrio entre a proteção antioxidante e as ER, pode ser chamado de equilíbrio “redox”, e essencial para manter a homeostase das funções intracelulares (Halliwell, 2011). O comprometimento desses sistemas de defesa antioxidante, assim como a excessiva produção de ER pode levar à oxidação de estruturas celulares tais como lipídios, proteínas, DNA nuclear, assim como de organelas como a mitocôndria o que desencadeia o dano oxidativo (Gutteridge, 1994; Halliwell, 2011; Vaya, 2013).

Defesa antioxidante é caracterizada por antioxidantes enzimáticos e os antioxidantes não enzimáticos. Os antioxidantes enzimáticos, que desintoxicam o organismo vivo transformando espécies reativas de alta reatividade em subprodutos menos reativos, como exemplo, as enzimas superóxido dismutase (SOD) e catalase (CAT) (Kasala *et al.*, 2016). Os antioxidantes não enzimáticos compreendem a glutationa reduzida (GSH) e vitaminas os quais são responsáveis por neutralizar espécies reativas (Ferreira e Abreu, 2007).

1.1.2. Cicatrização cutânea

Alterações vasculares e celulares, proliferação epitelial, proliferação de fibroblastos, síntese de produção de colágeno, produção de elastina, proteoglicanos, e a revascularização são alguns dos eventos biológicos envolvidos no processo de cicatrização até a contração da lesão (Almeida-Lopes *et al.*, 2001; Busnardo e Biondo-Simões, 2010; Gobbato, 2010). Em uma lesão tecidual, após o comprometimento de vasos sanguíneos e do extravasamento de sangue, o organismo reage fazendo a vasoconstrição e a ativação da coagulação através da agregação plaquetária, o que limita esse extravasamento pelo tecido (Rocha, 2014).

As plaquetas além de liberar fatores de coagulação que facilitam a formação de um tampão estático e também liberam fator de crescimento, tais como derivado de plaquetas (PDGF), transformante β (TGF-β), transformante α (TGF-α), epidérmico (EGF), e de crescimento de células endoteliais (VEGF), proteínas que irão atrair migração das células (tanto da circulação, como das regiões adjacentes). Além disso, ocorre também a liberação de citocinas que propiciam eventos fisiológicos complexos que conduzem à reparação e regeneração de tecidos (Nurden e Nurden, 2008; Grivennikov e Karin, 2010; Burnouf *et al.*, 2013).

Estas irão estimular por diferentes mecanismos a expressão gênica de NF-κB, Stat 3 entre outros que irão controlar a expressão de citocinas e mediadores inflamatórios, servindo assim como ferramenta para o controle imune e inflamatório (Grivennikov e Karin, 2010). Além disso, o coágulo sanguíneo proporciona uma matriz provisória, que facilita a cicatrização. (Diegelmann e Evans, 2004; Singer e Dagum, 2008; Mendonça e Coutinho-Netto, 2009). O processo de reparação tecidual pode ser divido em fases que se interrelacionam: fase inflamatória, proliferativa e de remodelagem. Essas fases se sobrepõem de forma contínua, e ocorre a migração celular, vasodilatação, angiogênese, formação de tecido de granulação e deposição de matriz extracelular (Araújo *et al.*, 2010).

1.2. LASERTERAPIA

Várias técnicas farmacológicas, gênicas e fisioterapêuticas têm sido desenvolvidas e aprimoradas para auxiliar no processo de cicatrização de úlceras cutâneas (Corazza *et al.*, 2007). No âmbito da Fisioterapia a terapia a *laser* (*light amplification by stimulated of radiantion*) na modalidade de baixa intensidade tem sido utilizada como um promissor recurso para o tratamento de tais lesões (Avci *et al.*, 2013).

A terapia *Laser* de Baixa Intensidade (LBI) refere-se a fototerapia ou fotobiomodulação, que é a utilização de fótons em uma irradiação não-térmica, que irá proporcionar alterações na atividade biológica (Wagner, 2012; Avci *et al.*, 2013). Essa modalidade terapêutica foi introduzida na década 1980 (Mester *et al.*, 1985), onde foi observado a melhora da cicatrização após a aplicação do LBI de rubi a 1 J/cm^2. O LBI é defino quanto aos seus parâmetros, tais como energia, comprimento de onda, modalidade continua ou pulsada (podendo ir até 5000 Hz), intensidade (energia/área), dose (tempo de irradiação/área irradiada), técnica de aplicação (Moriyama *et al.*, 2005; Posten *et al.*, 2005; Costa *et al.*, 2010).

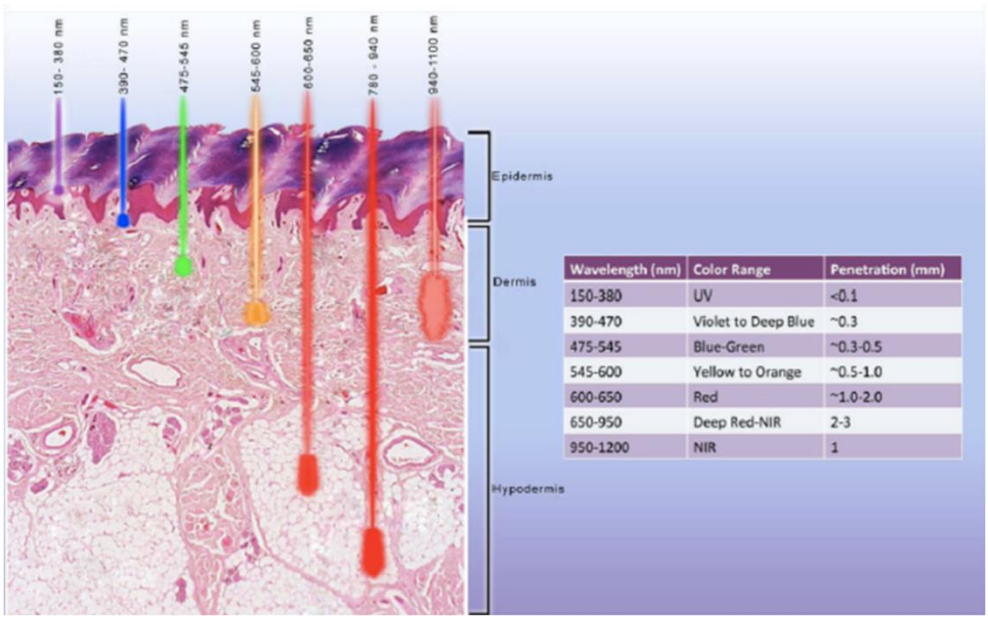
Estudos têm demonstrado a eficácia da irradiação com LBI com diferentes intensidades, em relação à aceleração do processo de cicatrização de diferentes tecidos e aumento da proliferação celular (Busnardo e Biondo-Simões, 2010; Laraia *et al.*, 2012), porém os mecanismos de ação do LBI ainda não estão completamente elucidados.

Efeitos da irradiação do LBI no tecido podem modificar atividades celulares, modular a transcrição de sinalização pró-inflamatoria e anti-inflamatória (Fukuda *et al.*, 2013; Hentschke *et al.*, 2013), dentre outras vias de sinalização celular, como a ativação do sistema antioxidante (Silveira *et al.*, 2007; Piva *et al.*, 2011). Além disso, a terapia LBI pode causar o aumento na respiração mitocondrial, na síntese de adenosina trifosfato (ATP) (Passarella *et al.*, 1984; Karu e Kolyakov, 2005), e divisão celular o que desta forma altera significativamente os processos metabólicos das células-alvo (Karu e Kolyakov, 2005; Avci *et al.*, 2013). A terapia a LBI tem sido bem relacionada também como efeitos anti-inflamatórios (Joensen *et al.*, 2012), na proliferação e migração celular (Liao *et al.*, 2014), aumento na produção de colágeno (Giuliani *et al.*, 2009; Pires *et al.*, 2011), aumento da angiogênese (Cury *et al.*, 2013), além dos efeitos analgésicos (Lins *et al.*, 2010). Entretanto atualmente o desafio para os pesquisadores é determinar o exato mecanismo de ação do LBI (Baldan *et al.*, 2012). Acredita-se que o mecanismo de ação biológico do LBI é através do

aumento da atividade do Citocromo C oxidase, pois esse é um cromóforo (Karu *et al.*, 2004; Karu e Kolyakov, 2005; Avci *et al.*, 2013).

Além disso, encontrar os parâmetros adequados para que a aplicação terapêutica seja mais eficaz, visto que existem diversos comprimentos de onda (nm) na laserterapia (fig. 3), e além disso, possibilidade de modificação de outros parâmentros, como modalidade, técnica de aplicação, dentre outros.

Figura 3 - Profundidade de penetração na pele dos vários comprimentos de onda (nm).



Fonte: Avci, P.; Gupta, A.; Sadasivam, M. et al. Low-level laser (light) therapy (LLLT) in skin: stimulating, healing, restoring. Seminars in cutaneous medicine and surgery; 2013: NIH Public Access.

O aumento do número de pacientes com úlceras cutâneas, ocasionadas por doenças crônicas degenerativas, instiga a busca de novos recursos a fim de melhorar a qualidade de vida destes indivíduos. Apesar da grande quantidade de pesquisas sobre a terapia LBI, ainda há grande carência de estudos que evidenciem os seus efeitos quanto ao estresse oxidativo, seus possíveis mecanismos de ação, e os melhores parâmetros de irradiação para a cicatrização. Assim, o uso de modelos animais de úlceras cutâneas associados ao tratamento com LBI permite investigar quais os melhores métodos e protocolos terapêuticos a serem empregados na clínica. Dessa forma, é possível estudar as alterações bioquímicas locais e sistêmicas, assim como morfológicas nos tecidos lesados em diferentes períodos de tempo, de acordo com as fases do processo inflamatório e cicatricial, e os efeitos do LBI 600nm com um

comprimento de onde de 20 J/cm^2 sobre estes parâmetros.

1.3. OBJETIVOS

1.3.1. OBJETIVO GERAL

Avaliar os efeitos da terapia a *laser* de baixa intensidade (LBI) sobre as alterações bioquímicas e morfológicas induzidas por um modelo de lesão cutânea em ratos.

1.3.2. OBJETIVOS ESPECÍFICOS

- Analisar a presença de células inflamatórias sistêmicas, alterações hematológicas e bioquímicas do sangue dos animais lesionados e tratados;
- Investigar os efeitos do LBI sobre o dano oxidativo na pele lesionada;
- Analisar os efeitos do LBI sobre os níveis de antioxidantes enzimáticos e não enzimáticos na pele;
- Avaliar os efeitos do LBI sobre as alterações histopatológicas da pele lesionada em diferentes momentos do processo de cicatrização tecidual;

2. MANUSCRITO

Low-Level Laser Therapy accelerates oxidative stress resolution and morphological recovery after a skin injury in rats

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Abstract

Ulcer(s) still exists as a pervasive problem and occurs in both hospital and community settings, which can contribute to further deterioration of a patient's general health condition. In ulcers occurs an extensive inflammatory response and overproduction of reactive oxygen species (ROS). Low level laser therapy (LLLT) has demonstrated benefit in wound healing accelerating the wound contraction. Therefore, the objective of this study was to analyze the LLLT effects in hematological changes and biochemical alterations in the skin, such as oxidative stress, antioxidant capacity, mitochondrial dehydrogenase activity and morphological changes induced by a skin injury model in rats, made through the instrument surgical "punch". For these studies rats were divided in Control, Sham, Lesion and Lesion+LLLT groups. The biochemical and morphological experimental analys were performed at different times (1st, 3rd, 7th, 14th, 21st day) after the development of injury in order to investigate the chronological development of the lesions and to verify the effectiveness of LLLT. Laser group showed an increase in total number of leukocytes, reduced DCF levels in comparison to values observed in 1^{st} day and reduced the TBARS levels in 7^{th} to 21^{th} days, when compared to control group. The MTT reduction levels and NPSH levels were increased in the 1st day after skin injury with treatment group. Considering data the LLLT accelerated skin tissue repair in a mechanism that involves increases of ROS production at early stages of tissue healing.

Key words: LLLT, skin lesions, inflammation, scarring.

Introduction

Ulcer still exists as a pervasive problem and occurs in both hospital and community settings, affecting all age groups, but mostly occurring among the elderly, the immobile, and those patients with severe acute illness or neurological deficits [1], which can contribute to further deterioration of a patient's general health condition [2].

Many factors contribute to the pathogenesis of ulcers. The main causes are chronic venous insufficiency, peripheral arterial occlusive diseases and diabetes. Some ulcers are caused by combinations of these well-known etiologic factors. In diabetic patients, distal symmetric neuropathy and peripheral vascular diseases are probably the most important etiologic factors in the development of ulcers. Less frequent causes of chronic ulcers are hematologic diseases, autoimmune diseases, genetic defects, infections, primary skin diseases, cutaneous malignant diseases, use of some medications and therapeutic procedures, and several exogenous factors [3-5]. Every year 2 to 3 million Americans are diagnosed with various types of chronic wounds. About 15% of older adults suffer from chronic wounds, including predominantly venous stasis ulcers, pressure ulcers (bedsores), and diabetic (neuropathic) foot ulcers, according to the Wound Healing Society [6] and the economic impact of the pressure sores treatment is astonishing [7].

Among the pathophysiological changes caused by skin ulcers are the structural changes in blood capillaries. It is widely accepted that ischemia is a primary factor in the etiology of pressure ulcers [8]. The hypothesis proposes that a mechanical loading of the tissue alters the arterial blood vessels, thereby causing local ischemia [8, 9]. The reintroduction of oxygen (O_2) in ischemic tissue by reperfusion causes cell death, since that have been shown that reperfusion harms the tissue more than the actual ischemia [10, 11]. After reperfusion has occurred, an extensive inflammatory response in the ischemic tissue could be expected determining a massive release of inflammatory mediators which, in turn, results in the overproduction of cytotoxic reactive oxygen species (ROS) [12, 13]. The ROS generation over the antioxidant defenses capacity to scavenging them leads to impairments of cellular biomolecules in a mechanism known as oxidative stress[14].

The ulcers healing process is complex and involves various biological events to the healing of injured tissues. Low level laser therapy (LLLT), which is widely used in physical therapy to treat a variety of skeletal muscle disorders [15], has demonstrated benefit in wound healing accelerating the wound contraction by increasing the fibroblast proliferation and epithelium proliferation and also ameliorating the synthesis of collagen [16]. One of the highlighted mechanism of LLLT action is via absorption of light by cellular mitochondria and

hemoglobin [17]. Houreld *et. al*. [18] showed in fibroblast an increase in cytochrome c oxidase activity as well as an increase in ATP synthesis, related with absorption of light by mitochondria. Moreover LLLT act as an inductor of tissues revascularization by increasing the levels of angiogenic factors, and, therefore, promoting a reduction in the area of necrosis [19, 20].

Therefore, the objective of this study was to analyze the effects of the LLLT 660 nm in some markers of oxidative stress and histopathological analysis of skin tissue, in different times of the inflammatory process and the ulcers healing process.

Materials and methods

Animals

Seventy male adult Wistar rats weighing 250 to 300 g were obtained from the Animal Breeding Unit of the Federal University of Santa Maria (UFSM). Animals were allocated in cages with food and water available ad libitum and maintained under standard conditions of temperature (22 ± 1° C) and illumination (12-h light / dark cycle). The study protocol followed the ethical rules established by the Guide for Care and Use of Experimental Animals published by the National Institutes of Health (NIH publication no. 85-23, revised in 1996). All procedures outlined in this study were approved by the Ethics and Research Committee UFSM (002/2014)

Experimental design

Rats were divided into two experimental groups (n=35), where the animal were subjected to two subgroups Lesion and Control, or Lesion + LLLT and Sham. The animals were anesthetized with an intraperitoneal injection of ketamine (10%) and xylazine (2%) administered as 0.1 mL of solution per 100 g of body weight.

The dorsal region of each animal was shaved and disinfected with alcohol 70%. In the left, between the infrascapular line and the tail, a circular area of the skin approximately 15mm diameter and 5mm deepening was removed with a punch in group: Lesion and Lesion+LLLT. The wounds were uniform in diameter, depth, and location. In the right was control integrity and radiation, group: Control and Sham, respectively. Were also anesthetized, shaved and disinfected with alcohol 70% to ensure standardization [21]. After the skin lesion, they were sub-divided in five subgroups according the analysis performed at 1, 3, 7, 14 and 21 days after injury.

Laser application

Aluminium gallium indium phosphide (Al-Ga-In-P) laser (Ibramed, Amparo, SP , Brazil) with a wavelength of 660mn, was applied in a single-point transcutaneous method with an energy density of 20 J/cm^2, 30mW power and a time of 40s. The first LLLT section was performed immediately after wounding the skin and was reapplied every day until the rats euthanasia.

Biochemical analysis

Rats submitted to the described experimental procedures were euthanized with a lethal administration of thiopental (120mg/kg *i.p.*) at 1, 3, 7, 14, or 21 days after injury. Immediately after euthanasia a sample of whole blood was collected by cardiac puncture in serum-separating tubes (5 mL) and another one in tubes containing EDTA (5 mL) (Vacutainer®).

A sample of skin of each animal (1cm around the wound margin and deeply to the subcutaneous fascia) was surgically excised and frozen in liquid nitrogen (-80 °C) until the biochemical analysis. One sample was used for histology.

Skin homogenates were prepared in cold saline (NaCl 0.9%) in 1:5 (weight:volume) proportion using Ultra stirrer X80. Homogenates were centrifuged at 2,000rpm for 10 min to yield the low-speed supernatant (S1) fractions that were used for analysis.

Hematologic analysis

Serum samples were obtained after whole blood centrifugation at 3000 rpm for 15 minutes) and were stored into Eppendorf® plastic tubes at -20ºC until quantification of albumin, total protein (TP) and total globulin (TG) levels. These analyses were carried out using Labtest Kits (Labtest Diagnóstica SA) through the automatic analyzer CELM SBA 200® (CELM, Barueri/SP, Brazil)

Moreover, the whole blood samples collected into tubes containing EDTA (Vacutainer®) were used to determine total leukocytes (WBC), total erythrocytes (RBC), hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and platelets levels using an automatic counter COULTER T890® (Coulter Electronics, Inc, Hialeach, FL, USA). Blood smears were fixed in methanol and stained with Instant-Prov (NewProv®) stain for determination of

the differential WBC count. At least 200 WBCs were counted for differential WBC determinations.

Measurement of lipid peroxidation (LPO)

LPO was quantified by malondiadehyde (MDA) levels formation in skin homogenates [22]. In summary, skin homogenates were incubated in a medium containing Tris-HCl as a buffer (10mM, pH 7.4) for 60 minutes. Incubation was ended by acetic acid buffer (pH 3,6) followed by lauryl sodium sulphate (SDS 8,1%) and thibarbituric acid (TBA 0,6%, pH 6,2) addition. The mixture was led to color reaction (60 min. at 100ºC), and thiobarbituric acid reactive substances (TBARS) levels were spectrophotometrically measured at 532 nm using a standard curve of MDA.

Measurement of reactive oxygen species (ROS) production

ROS production in skin homogenates was determined by oxidation of reduced dichlorofluoresceine (H_2DCF-DA) [23]. Briefly, skin homogenates were added to standard medium containing Tris-HCl as a buffer (10mM, pH 7.4) and H_2DCF-DA (1mM) for 60 minutes in a condition without light. Fluorescence quantification was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm, in a spectrofluorimeter,using oxidized diclorofluoresceine (DCF) as a standard.

Measurement of mitochondrial dehydrogenase activity (MTT reduction assay)

This assay is based on the ability of mitochondrial enzymes to metabolize MTT into formazan, a reaction that takes place only in functionally intact mitochondria, was determined by methyl tetrazolium salt (MTT) reduction [24]. Briefly skin homogenates were incubated with MTT at 30ºC for 60min. Dimethyl sulphoxide (DMSO 1mL) was added to extract colored components and measures were made at 570 nm. Results were expressed in percent of the control (skin samples without injury and without LLLT) values.

Measurement of antioxidant enzyme activities

The antioxidant enzymes total superoxide dismutase (SOD) and catalase (CAT) were measured in skin homogenates as described previously [25, 26]. In SOD analysis, the skin homogenate was added to a medium containing ethylenediamine tetraacetic acid (EDTA 2mM) and bicarbonate buffer ($NaHCO_3$/ Na_2CO_3 50 mM, pH 10.3). Epinephrine (4mM) was added to start the kinetic of SOD activity during 5 minutes. The colored product of

epinephrine degradation (that was inhibited by cellular SOD activity) was spectrophotometrically measured at 480nm. The SOD enzyme activity was expressed in units of the enzyme activity per milligrams of protein. In CAT assay, skin homogenate was added to a medium containing potassium phosphate buffer (50 mM KH_2PO_4, 50 mM K_2HPO_4; pH 7.4). The kinetic analysis of CAT was started after hydrogen peroxide (H_2O_2 1 mM) addition. The CAT activity was determined using the molar extinction coefficient 36 $M^{-1}cm^{-1}$ and the reaction was spectrophotometrically measured at 240 nm.

Non-protein thiol (NPSH) levels measurement

NPSH levels were determined in skin homogenates according to the method proposed previously [27] with some modifications. Briefly, homogenate samples were precipitated with TCA (5%) (1:1) and subsequently centrifuged at 4,000rpm for 10 min. Supernatant fraction (500 μL) was added to a reaction medium containing K-phosphate buffer (0.25mM, pH 7.4) and DTNB (1 mM). Spectrophotometrical measures were made at 412 nm. Results were calculated in relation to a standard curve constructed with GSH (glutathione reduced) at known concentrations and also corrected by the protein content

Histopathological analysis

One sample of the skeletal muscle tissue was used for the histopathological analysis in order to investigate microscopic changes in the normal tissue structure. After being excised, the skin was maintained in buffered formaldehyde solution (10%) until the microscopic preparation and colorization. The skin samples were sectioned transversely, the histological slides were stained with hematoxylin and eosin and then submitted to the histopathological analysis.

Protein levels measurement

Protein content was determined using bovine serum albumin (BSA) as standard according to [28]. Spectrophotometrical measures were made at 595nm.

Statistical analysis

Statistical analysis was performed using GraphPad (version 5.0 for Macintosh OSX, GraphPad Software, San Diego, CA). Significance was assessed by two-way analysis of variance (ANOVA), followed by Bonferroni Test for post-hoc comparison. Values of $p<0.05$ were considered statistically significant.

Results

Effects of LLLT in blood tissue

LLLT did not change the levels of total proteins, albumin and total globulins (Table 1). On the other hand, was observed a significant increase in total number of leukocytes (WBC cells) after 1 to 3 days of LLLT in comparison to control (untreated) conditions (Table 2) ($p<0.05$).

Effects of LLLT on skin oxidative damage

Skin injury induced a significant increase in ROS generation from the 1st to the 7th day after skin injury untreated. Furthermore, LLLT reduced the DCF values in Sham group after 3 days of treatment. From the 3rd to 21th day, LLLT reduced DCF levels in comparison to values observed in 1 day Lesion+LLLT. The reduction of Lesion DCF values occurred only after 14 days after skin lesion onset (Figure 1A) ($p<0.05$).

Lipid peroxidation was also significantly increased from the 1st to the 7th day after skin injury in Lesion group. However, LLLT reduced the TBARS values to control (uninjured) conditions only after 7 days of treatment. Moreover, LLLT significantly reduced the TBARS levels from 3rd to 21th days of treatment in comparison to values observed in 1 day in the Lesion+LLLT (Figure 1B) ($p<0.05$).

Effects of LLLT on mitochondrial dehydrogenase activity

Figure 2 shows that LLLT increased the dehydrogenase activity starting in 1st day until 7th day after injury ($p<0.05$).

Effects of LLLT on skin enzymatic and non-enzymatic antioxidants

SOD enzyme activity levels were significantly increased from the 1st to the 3rd day after the skin injury in Lesion group, and Lesion+LLLT did not change this increase. Sham determined an isolated increase in SOD activity 3 days after the start of treatment (Figure 3) ($p<0.05$).

CAT enzyme activity was not significantly changed by skin lesion or LLLT in any one of the analyzed days (data not show).

Sham demonstrated a significant increase in NPSH levels in the 1st day, and from the 1st to 3rd days after skin injury in the Lesion+LLLT group. Increased NPSH were observed also 3 days after skin lesion in untreated animals (Figure 4) ($p<0.05$).

Effects of LLLT on skin histopathology

An increased infiltration of inflammatory cells was observed in the 1st and 3rd days after skin injury onset associated with LLLT. Moreover, in the 7th day, changes in density and activation of fibroblasts, keratinization in surface of wound, presence of collagen fibers and partial re-epithelization were observed. Presence of the collagen fibers and complete re-epithelization occurred just in 14th day after LLLT (Figure 5).

In Lesion conditions an increased infiltration of inflammatory cells was observed only 3 days after skin injury. In the 7th day we observed granulation tissue composed of a proliferation of blood vessels and fibroblasts and partial re-epithelization. A complete re-epithelization was observed just in 14th day (Figure 5).

Discussion

In the present study, we evaluated the effects of LLLT on the skin healing by measuring some biochemical markers of oxidative stress and hematological indicators of the inflammatory process. Such findings, we can observe the increased generation of reactive species at first tissue repair, and raise of the levels antioxidants after irradiation with LLLT in the injured tissue as in the intact tissue. Recent studies have shown that LLLT acts in the mitochondrial, transport chain electron specifically [29]. Therefore, increases the importance of investigating the oxidative response in these tissues treated.

The analysis of biochemical and hematological indicators revealed that the effects of LLLT were observed only in the number of leukocytes. In fact, LLLT increased WBC levels when compared to control until the 3rd day after the treatment beginning (Table 2). This result is in accordance to the observed by Boschi et al.[30] in a study using LLLT 21J/cm^2 (InGaAlP) in 1, 2, and 3 hours after induction inflammation by carrageenan (0.2 ml). Moreover, Machneva et al. [31] also tested three different types of lasers: HeNe laser (red, 632.8 nm), the solid-state laser (green, 532.5 nm), and the HeCd laser (blue, 441 nm); the authors reported an increase of the leukocytes activity in the red laser radiation with a dose up to 1.5 J/cm^2 only in the groups intraperitoneally injected with lipopolysaccharide (LPS). Therefore, our findings demonstrated that LLLT increased significantly the leukocytes levels, characterizing the skin repair and healing.

In general, our results showed that LLLT associated with lesion raised the reactive species levels at the first moments of wound healing process. Previous studies already related LLLT with an increased electron flue through the mitochondrial transport chain [29, 32, 33], which could lead to an increased electron leakage and, consequently, ROS generation. As a

result, modified cellular activities could be expected, such as the activation of the cellular antioxidant system[32-34], changing the metabolic processes of target cells such as the inflammatory response and scarring process[35, 36]. However, after 7 days of irradiation it is possible to observe a decrease in ROS and TBARS levels (fig.1). These results suggest that LLLT can use as a cellular signaling pathway the increases oxidative, to thereby decrease the time needed for tissue repair.

The specific mechanisms of the photostimulatory effects are not well understood, but researches have shown effects at molecular, cellular, and tissular levels. Previous study already reported evidence that LLLT can increase ATP synthesis after 3 min of irradiation [37]. Karu et al.[38] showed also increases in ATP synthesis induced by LLLT in HeLa (human cervical cancer) cells, but only after 20-25 min of irradiation. Other studies, have reported LLLT effects on mitochondria, such as also increases in membrane mitochondrial potential (MMP), protect against oxidative stress[39] and in mitochondrial complex IV (cytochrome c oxidase) activity in a dose-dependent manner[18]. In fact, the activation of mitochondrial complex IV is thought to be the basic biological mechanism behind the effects of LLLT because it constitute the major site of light issued by LLLT absorption[40-43].

Taken together, these effects of LLLT on mitochondrial activity could also explain the increases in MTT levels observed from the 1st to the 7th day after LLLT beginning in uninjured conditions (Figure 2). These results are in accordance to those observed by Volpato et al. [44], which noticed a positive effect (increased MTT levels) in treated fibroblasts 72 hours after LLLT. Since that an increased mitochondrial function could mean a significant index of cells viability, we propose that the photobiomodulatory interference produced by LLLT probably acts improving the cell survival and proliferation after a skin injury.

On the other hand, the effects of LLLT on SOD activity reinforce the hypothesis of its action on cellular systems. In fact, the expected high levels of electron escape from mitochondrial transport chain determined by LLLT could result in augmented levels of the ROS superoxide anion (O_2^-), which is a substrate for SOD activity. Therefore, we propose that the observed increases in SOD activity could mean a compensatory response of injured cells to trigger the high O_2^- production related to the oxidative damage in injured area. Furthermore, the effect of LLLT in Sham group 3 days after treatment start could represent a compensatory response to increases of mitochondrial activity and consequently of O_2^- exit. Moreover, Silveira et al.[15] found decreases in SOD activity 5 days after LLLT while we observed this pattern only after 7 days of treatment. Besides, showed an increase in the levels of the SH no-protein, triggered by the application of LLLT, because the increase was

significant in both groups that received the irradiation (Sham and Lesion+LLLT).

In our study, the histopathological analysis revealed an increase in the density and activation of fibroblasts, in the collagen deposition, and skin re-epithelization in LLLT group, demonstrating that 660nm lights irradiation could significantly enhance skin injury healing. Similar results by other studies in which He–Ne laser at 632.8nm and 660–670 nm with a fluence of 1–6 J/cm^2 stimulated healing in normal and impaired wounds (burns and diabetic), increasing re-epithelialization and granulation tissue formation[29, 45, 46]

Therefore, we observed that the treatment with LLLT after injury promotes tissue repair, re-establishes the normal histopathological parameters in skin tissue. However, it shows effects compared with Control and Sham groups, increasing the ROS generation and lipid peroxidation.

Conclusion

In conclusion we suggest that LLLT accelerated skin tissue repair in a mechanism that involves increases of ROS production at early stages of tissue healing. Considering that ROS has a signaling role for cells survival and proliferation, we hypothesize that LLLT anticipates the oxidative changes in skin tissue after an injury and also abbreviates the time needed to its resolution. In order to confirm this hypothesis, studies of LLLT effects on mitochondria activity and signal transcription of skin cells are needed to elucidate its mechanism of therapeutic action and also to amplify and improve LLLT employment by clinicians.

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Conflict of Interest statement

The authors declare no conflicts of interests.

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Figure captions

Table 1. Effect of LLLT treatment on biochemical and hematological indicators. Data are expressed as mean ± SEM. Significance was assessed by two–way analysis of variance (ANOVA), followed by Bonferroni test for post hoc comparison # indicates significant differences in comparison to respective control day values ($p < 0,05$); * indicates significant differences in comparison to values of the day 1in the same experimental group($p < 0,05$).

Table 2. Effect of LLLT treatment in the blood red series and blood white series. Data are expressed as mean ± SEM. Significance was assessed by two–way analysis of variance (ANOVA), followed by Bonferroni test for post hoc comparison # indicates significant differences in comparison to respective control day values ($p < 0,05$); * indicates significant differences in comparison to values of the day 1 in the same experimental group($p < 0,05$).

Fig 1. Effects of LLLT (660nm) on skin oxidative damage. In 1A the levels of ROS generation were expressed in nmol of DFC per mg of protein. In 1B the levels of lipid peroxidation were expressed in nmol of MDA per mg of protein Significance was assessed by two–way analysis of variance (ANOVA), followed by Bonferroni test for post hoc comparison * indicates significant differences in comparison to respective control day values ($p < 0,05$); # indicates significant differences in comparison to values of the day 1 in the same experimental group($p < 0,05$).

Fig 2. Effects of LLLT (660mn) on skin cells viability. MTT reduction levels were expressed in percentage of absorbance control values. Significance was assessed by two–way analysis of variance (ANOVA), followed by Bonferroni test for post hoc comparison * indicates significant differences in comparison to respective control day values ($p < 0,05$); # indicates significant differences in comparison to values of the day 1 in the same experimental group($p < 0,05$).

Fig 3. Effects of LLLT (660mn) on skin antioxidant enzyme activities. SOD values were expressed in units of the enzyme activity per mg of protein. Significance was assessed by two–way analysis of variance (ANOVA), followed by Bonferroni test for post hoc comparison * indicates significant differences in comparison to respective control day values

($p < 0,05$); # indicates significant differences in comparison to values of the day 1 in the same experimental group($p < 0,05$).

Fig 4. Effects of LLLT (660mn) on skin antioxidant. SH no-protein values were expressed in units of the enzyme activity per mg of protein. Significance was assessed by two–way analysis of variance (ANOVA), followed by Bonferroni test for post hoc comparison * indicates significant differences in comparison to respective control day values ($p < 0,05$); # indicates significant differences in comparison to values of the day 1 in the same experimental group($p < 0,05$).

Fig 5. Effects of LLLT (660mn)in Histopathology analyses. Control (A,B,C,D), Lesion (E,F,G,H), Sham (I,J,K,L), Lesion+LLLT (M,N,O,P).

Table 1.

	Protein (mg/dL)		Albumin (mg/dL)		Globulin (mg/dL)	
	Control	LLLT	Control	LLLT	Control	LLLT
Day 1	7.03± 0.44	6.6± 0.23	3.03± 0.14	2.9± 0.02	4.0± 0.3	3.7± 0.23
Day 3	7.0± 0.09	6.5± 0.15	2.9± 0.12	2.8± 0.16	4.0± 0.2	3.6± 0.03
Day 7	7.2± 0.11	6.3± 0.42	2.7± 0.08	2.5± 0.15	4.4± 0.03	3.8± 0.27
Day 14	6.4± 0.26	6.0± 0.03	2.8± 0.06	2.8± 0.06	3.3± 0.20	3.2± 0.05
Day 21	5.4± 0.11*	6.0± 0.05	2.7± 0.08	3.0± 0.03	2.6± 0.03*	2.9± 0.03

Table 2

	Day 1		Day 3		Day 7		Day 14		Day 21	
	Control	LLLT	Control	LLLT	Control	LLLT	Control	LLLT	Control	LLLT
RBC (x 10^6/ µL)	7.0±0.68	5.19±2.03	5.64 ±1.34	7.04±0.53	6.0±0.69	6.32±0.21	6.14±0.51	5,97±0.44	7.26±0.03	7.43±0.16
HGB (g/dL)	7.0±0.68	5.19±2.03	5.64 ±1.34	7.04±0.53	10.23±1.21	11.76±0.31	6.14±0.51	5.97±0.44	7.26±0.03	7.43±0.16
HCT (%)	37.0±3.56	37.6±0.85	29.76±6.97	37.53±2.22	31.66±3.38	34.66±0.66	32.5±2.65	31.6±2.35	39.23±0.03	40.3±0.80
VCM (fl)	53.43±0.26	53.06±0.95	52.93±0.29	53.46±1.0	52.0±0.1	52.33±0.33	54.1±0.55	52.2±0.8	54.0±0,2	54.0±0.12
CHCM (%)	33.53±0.23	33.43±0.16	33.8±0,75	33.3±0.26	34.15±0.37	34.8±1.62	33.86±0.56	34.33±0.59	33.9±0.34	33.35±0.26
WBC (x 10^3/µL)	10.06±0.78	14.76±0.39#	5.23±0.50	7.83±1.88#	6.43±0.23	7.4±0.66	8.06±1.32	7.76±2.10	9.53±0.77	8.96±0.54
Neutrophils (%)	54.0±6.55	66.0±6.02	75.33±6.22	62.33±4.97	51.33±6.06	61.33±2.18	81.16±3.08	72.66±6.74	61.0±1.15	68.33±0.88
Lymphocytes (%)	46.66±5.89	61.66±6.66	71.0±6.65	55.66±4.05	44.5±5.48	58.0±1.52	76.33±2.33	63.66±8.45	59.0±1.15	62.66±0.88
Eosinophils (%)	3.66±0.88	0.33±0.33#	1.0±0.57	1.0±0.57	2.0±0.22	1.33±0.33	3.0±1.0	4.0±1.15	0.66±0.33	1.0±0.42
Monocytes (%)	3.66±0.33	4.0±1.0	3.33±1.27	5.66±0.88	5.0±0.57	2.0±0.57	2.0±0.57	5.0±0.58	1.66±0.33	5.0±0.1
PLT (x 10^3/µL)	758.33±93.3	566.33±45.3	384.0±42.0	381.66±58.6	538.66± 27.4	488.33±13.8	746.66±62.6	597.66±58.6	679±2.60	654.33±11.8

Figure 1

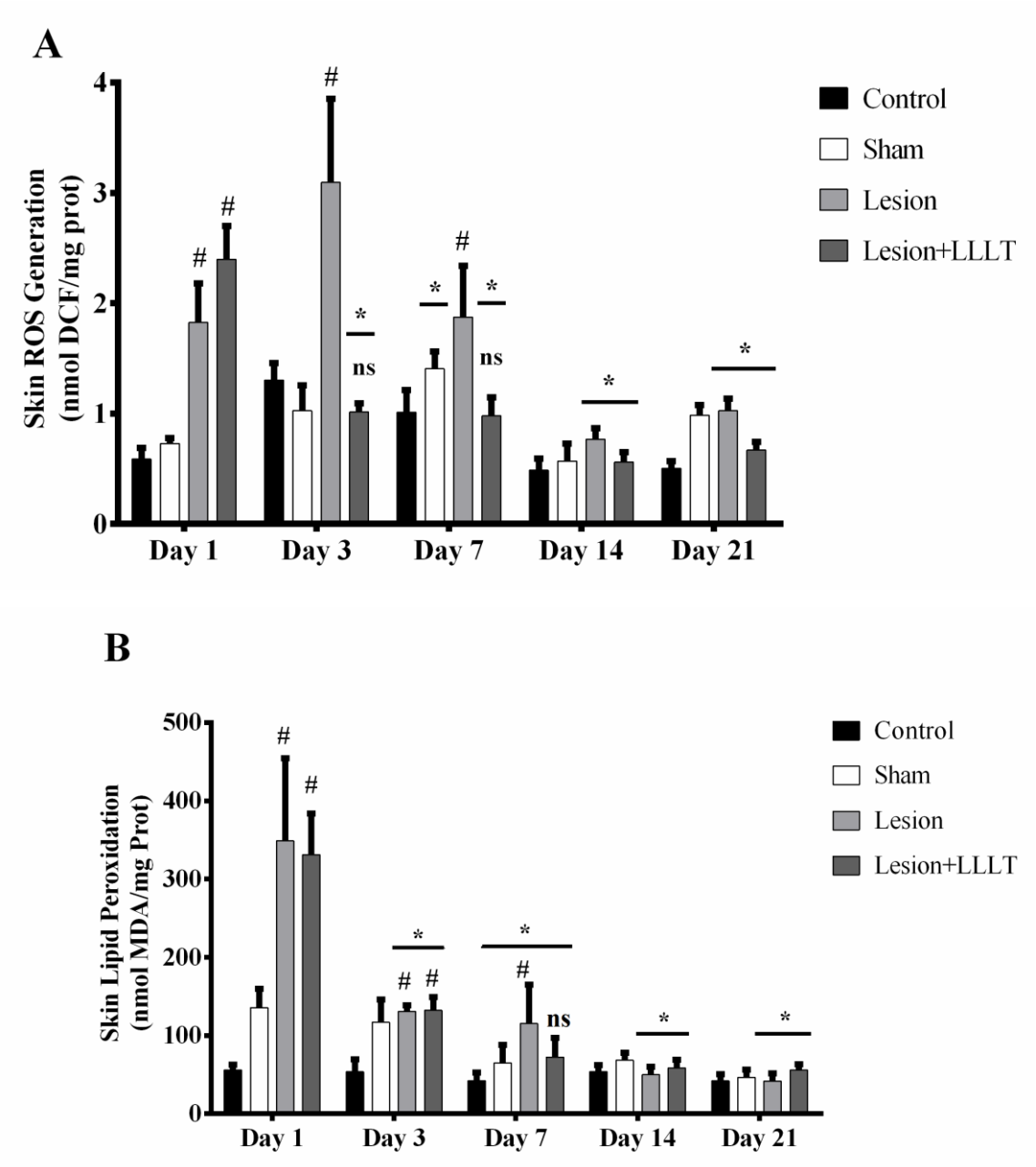

A
Skin ROS Generation
(nmol DCF/mg prot)
0
1
2
3
4
Day 1
Day 3
Day 7
Day 14
Day 21
Control
Sham
Lesion
Lesion+LLLT
#
*
ns
B
Skin Lipid Peroxidation
(nmol MDA/mg Prot)
0
100
200
300
400
500
Day 1
Day 3
Day 7
Day 14
Day 21
Control
Sham
Lesion
Lesion+LLLT
#
*
ns

Figure 2

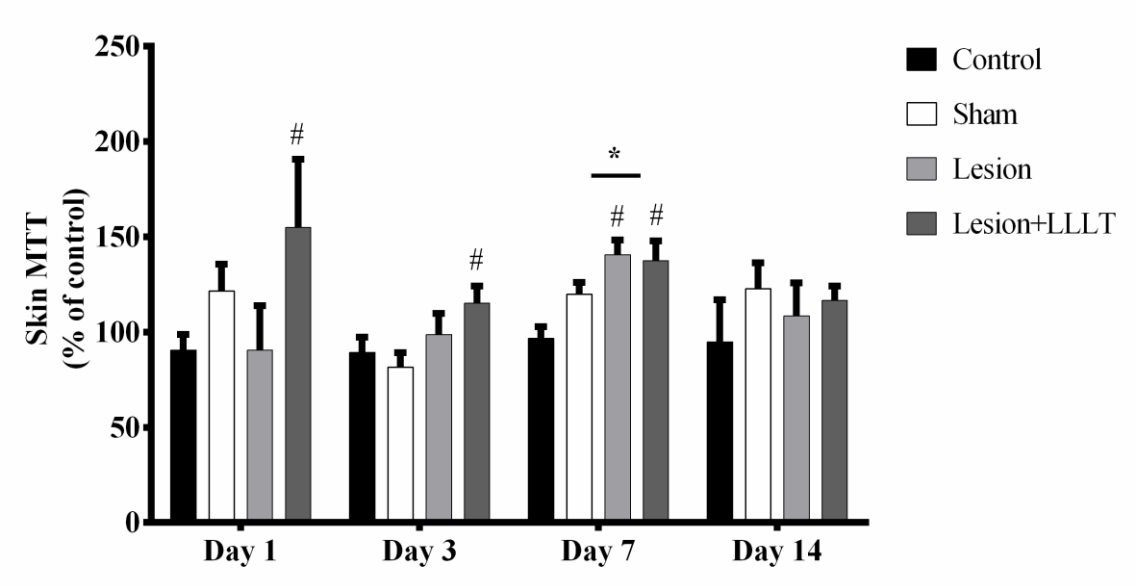

Skin MTT
(% of control)
250
200
150
100
50
0
Day 1
Day 3
Day 7
Day 14
#
#
*
#
#
Control
Sham
Lesion
Lesion+LLLT

Figure 3

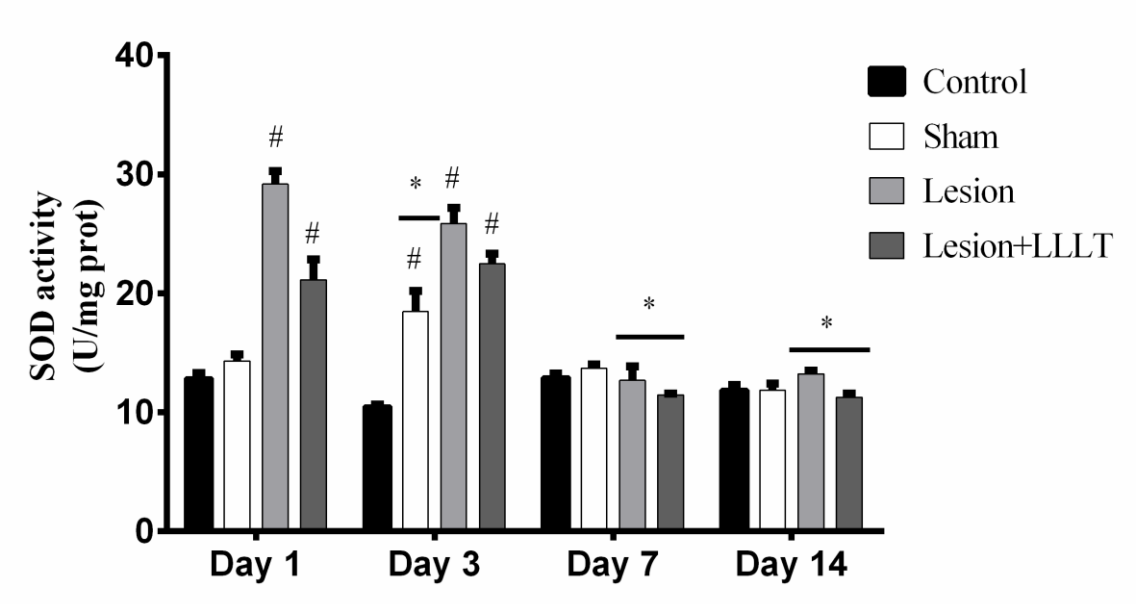

SOD activity
(U/mg prot)
40
30
20
10
0
Day 1
Day 3
Day 7
Day 14
Control
Sham
Lesion
Lesion+LLLT
#
*
#
#
#
*
*

Figure 4

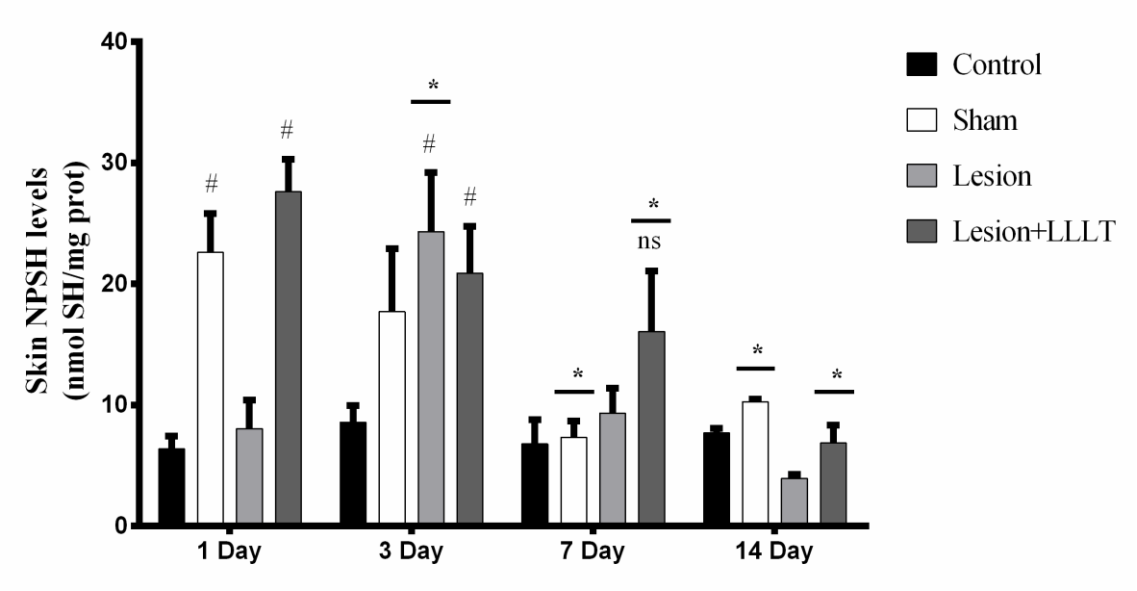
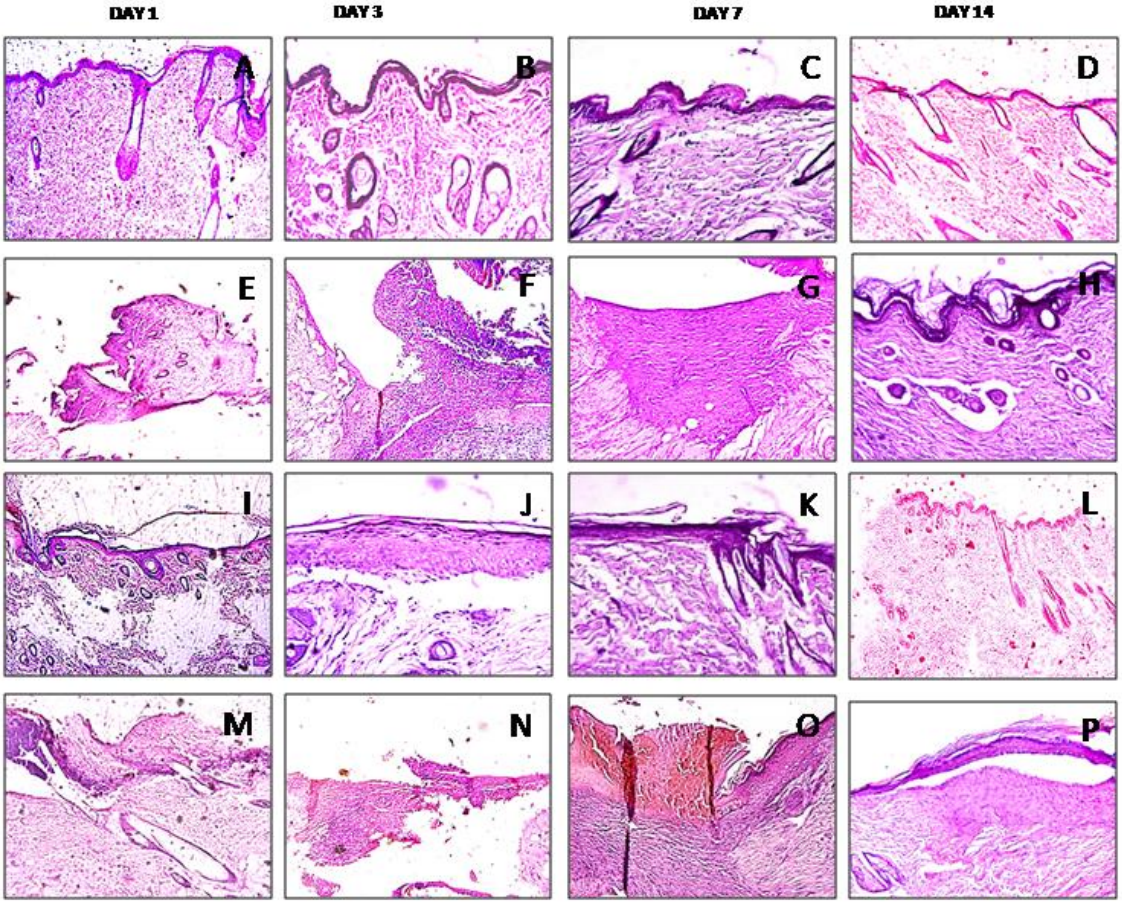

Skin NPSH levels
(nmol SH/mg prot)
40
30
20
10
0
1 Day
3 Day
7 Day
14 Day
#
*
ns
Control
Sham
Lesion
Lesion+LLLT

Figure 5



3. CONCLUSÃO

Os resultados demonstrados neste trabalho elucidam o potencial terapêutico do Laser de Baixa Intensidade (LBI) para o tratamento de lesões cutâneas, mesmo com o aumento da produção de espécies reativas de oxigênio (EROs) no estágio inicial da cicatrização.

Esse aumento pode estar envolvido no mecanismo de sinalização celular, que irá proporcionar às células de sobrevivência e proliferação, já que as EROs tem um papel importante na sinalização celular, podendo assim está diretamente envolvido na regeneração do tecido tratado com LBI.

Podemos comprovar que a terapia empregada com LBI antecipa as alterações morfológicas da pele após uma lesão necessária para sua cicatrização, abreviando o tempo necessário para a sua total cicatrização.

Para confirmar esta hipótese, são necessários estudos sobre os efeitos de LBI sobre a atividade de mitocondrial, e a transcrição de sinalização celular a fim de elucidar por completo seu real mecanismo de ação terapêutico.

4. PERPECTIVAS

A partir dos resultados obtidos, mais estudos da são necessários para identificar precisamente o mecanismo de ação da Terapia Laser de Baixa intensidade (LBI) na regeneração tecidual, como vias de sinalização celular envolvidas no reparo tecidual e sobrevivência celular, que podem estar sendo acionados por essa irradiação terapêutica. E a atividade mitocondrial desses tecidos tratados, permeabilidade de membrana mitocondrial, atividade de complexos mitocondriais, sua funcionalidade como um todo.

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